AZOFICATION

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REPRINTED FROM
SOIL SCIENCE, VOL. VI, NO. 3, SEPTEMBER, 1918

Reprinted from Soil Science,
Vol. VI, No. 3, September, 1918

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Received for publication August 14, 1918

The maintenance of the nitrogen supply of the soil is the phase of soil fertility which has received greatest consideration both from the scientist and from the practical agriculturist. Nitrogen is one of the more expensive commercial fertilizers and is, in the majority of soils, the limiting factor of crop production. The supply of combined nitrogen on the earth is comparatively small and it is possible to calculate approximately the time necessary for its exhaustion. Basing his conclusion on such a calculation, at least one scientist has predicted dire calamity to the human race were science not able soon to solve this problem. Science has measured up to its requirements in this regard, for the synthetic production of combined nitrogen has been accomplished, and this in a manner so highly satisfactory that it is able to compete successfully with the product of natural deposits. Advancements have also been made in our knowledge of the underlying principles influencing the natural processes which govern the fixation of nitrogen in the soil. Although there is much yet to be learned in this field it is upon the control of these natural processes that ultimate success will be based.

It has been known for generations that uncropped soils increase in fertility. Less ancient, however, is the knowledge that this increase may be due to a gain of nitrogen in the abandoned soils. Even more recent than this is the knowledge that it may be due to bacteriological action.

In the middle of the nineteenth century Boussingault (19) wrote: "Vegetable earth contains living organisms—germs—the vitality of which is suspended by drying and reestablished under favorable conditions as to moisture and temperature." He also hinted at the fact that these microorganisms take part in the process of nitrogen fixation. He spread out thinly 120 gm. of soil in a shallow glass dish and for three months moistened it daily with water free from nitrogen compounds. At the end of this time analysis showed that it had lost carbon, but had gained nitrogen. It was not until thirty years later that Hellriegel and Wilfarth made their discovery of nitrogen-fixation by symbiotic organisms. At that time the laboratory technique of modern bacteriology was still undeveloped. Since then, however, we have learned much concerning the relationship of plants to free and combined nitrogen of the air and of the soil. We know that soil gains in nitrogen are often due to microorganisms, either living free in the soil or in company with the

higher plants. The production of nitrogen compounds out of atmospheric nitrogen by bacteria independent of higher plants is designated non-symbiotic nitrogen-fixation, or azofication. When fixation is accomplished by bacteria living in connection with and receiving benefit from higher plants, it is considered symbiotic nitrogen-fixation.

As early as 1883 Berthelot (13) undertook the study of soils with regard to their relation to free and combined nitrogen, and as a result of these studies he was the first definitely to recognize that gains which occur in bare unsterilized soils are due to microscopic organisms. He found that when 50 kgm. of arable soil were exposed to air and to rain in a vessel for 7 months, after allowing for the small amount of combined nitrogen brought down by the rain, there was a gain in nitrogen of more than 25 per cent. In another experiment in which the soil was first washed free from nitrates, there was a gain of 46 per cent. Many other experiments showed gains from 10 to 15 per cent. Berthelot was not content with the bare knowledge that nitrogen is fixed in the soil by living organisms, but continued his work with the idea of isolating some of these organisms. With the aid of Guignard, he made soil inoculation into sterile bouillon and from this prepared gelatin plates. Cultures were taken from the colonies growing on the plates and bacteria were tested for their nitrogen-fixing power. His results were conclusive that there exist within the soil chlorophyll-less bacteria capable of fixing atmospheric nitrogen. His work had shown that these organisms act best at summer temperatures, between 50° and 104° F., in the presence of a good supply of oxygen, a proportion of water in the soil not exceeding 12 to 15 per cent and not falling below 2 to 3 per cent. They require carbon, hydrogen and enough combined nitrogen to promote initial growth. The nitrogen, gained by the soil was proteinaceous in nature, being insoluble in water. Although some of his soils had gained large quantities of nitrogen, he considered that the fixation of atmospheric nitrogen by microorganisms has its limits, since the organisms isolated drew from the atmosphere only so long as the amount fixed in the medium was not great. Heating the soil to 230° F. immediately stopped the process.

Prior to this a number of chemists, notably König and Kiesow (101), Armsby (1), Birner (14), Kellner (91), Deherain (35) and Avery (3) had found that when organic matter in one form or another undergoes fermentation there is frequently an increase of nitrogen in the fermenting substance. Armsby states it thus: "We must conclude that decaying organic substances in the presence of caustic alkali are able to fix free nitrogen without the gain being manifest as nitric acid or ammonia, and probably with the formation of these bodies." His explanation of the process was that the nascent hydrogen evolved during the fermentation process reacted with the free nitrogen of the air. Others considered that the active agents were compounds of iron, manganese, and lime existing in the soil and in some way acting as catalytic agents.

Berthelot's discovery interested Winogradski (209) who commenced work which eventually bridged the chasm. He employed, as a medium, a nutritive solution free from combined nitrogen, but containing mineral salts and dextrose. Fifteen separate species of soil bacteria were isolated, but only one—a long spore-bearing bacillus which developed normally in the absence of combined nitrogen and seemed to produce butyric fermentation—fixed nitrogen to any appreciable degree. Quantitative tests showed that the maximum fixation was attained where no combined nitrogen was purposely added, and that on the addition of such, fixation of nitrogen was diminished. For example, several determinations gave the following results:

N as NH_3 in dextrose solution	2.1	4.2	6.4	8.5	21.2
N fixed	7.0	5.0	5.5	3.6	2.2

The presence of combined nitrogen tends to decrease fixation. He concluded that in order for any gain to be made, the ratio of the combined nitrogen to the sugar should not exceed 6: 1000. Because of the characteristic formation of clostridia in his cultures, Winogradski named the organism *Clostridium pasteurianum*. The conclusion which the author reached, however, was that the power of fixing nitrogen is not general among microorganisms, but confined to a few special forms.

Following Winogradski, Caron (25) made some very interesting discoveries. He found that soils under leafy crops contain greater numbers of bacteria than those under grasses. He also observed that the bacterial flora of soils in the spring are different from those in the fall both quantitatively and qualitatively. He used in vegetation experiments pure cultures of the bacteria most frequently encountered in natural soils. Some soils were inoculated with bouillon culture, whereas others received only sterile bouillon. The crop yields were usually in favor of the inoculated plots, but showed variations from season to season. Exceptionally good results were obtained with a spore-bearing bacillus which he termed *Bacillus ellenbachensis*.

Caron's work led to the commercial exploitation of his cultures, one of which, "alinit," was the subject of much study and discussion. This culture was found to contain, according to Severin, two closely-related bacilli which he chose to designate as *B. ellenbachensis A* and *B.* These had the power to fix nitrogen to some extent. Tests with "alinit," however, have not confirmed to any great extent the claims of its exploiters.

In 1901 Beijerinck's (7) investigations led to an extremely important addition to the history of non-symbiotic nitrogen-fixation. He described a new group of large aerobic bacilli to which he gave the generic name *Azotobacter*.

In an early paper published by Beijerinck and Van Delden (9), they maintain that *Azotobacter* are incapable of fixing appreciable quantities of nitrogen in pure culture, but are dependent to a large extent on *Granulobacter, Radiobacter*, and *Aerobacter*. They considered that in mixed cultures the *Granulobacter, Radiobacter*, and *Aerobacter* possess the power of fixing nitrogen in the

presence of *Azotobacter*, which grows at the expense of the combined nitrogen escaping from them into the solution.

A little later Gerlach and Vogel (50) succeeded in isolating from soil the *Azotobacter* of Beijerinck and in showing that in pure cultures and in the presence of salts of organic acids, *Azotobacter* are capable of active nitrogen-fixation. They obtained a fixation of 9 mgm. of nitrogen in a 1 per cent solution of grape sugar. But Beijerinck challenged this assertion claiming that their cultures were not pure but were mixed with other forms difficult to separate. The claims of Gerlach and Vogel were substantiated by the work of Freudenreich (47), Koch and Lipman (122). The latter not only showed that the *Azotobacter* possess the power of fixing nitrogen in pure cultures, but he explained the failures recorded by others.

Although not necessary, the presence of other organisms often proves advantageous (47). Lipman (122) found that in the presence of such forms as *B. radiobacter* and *B. levaniformus* the nitrogen-fixation is faster and goes on at a more regular rate.

To the two species of *Azotobacter*—*A. chroococcum* and *A. agilis*—described by Beijerinck and Van Delden, Lipman (122, 123) added *A. vinelandii*, *A. beijerinckii*, and *A. woodstownii*. Later Löhnis and Westermann (134) described *A. vitreum*, and after a study of 21 cultures of various *Azotobacter* concluded that they represented only four types. *A. chroococcum* is most widely distributed in the soils so far studied.

The discussion of the subject thus far has been more or less confined to the *Azotobacter*, but investigations of Beijerinck and Van Delden (9), Löhnis (127), Moore (144), Chester (26), Bredemann (20) and others (168) have brought to light other microorganisms having the power to fix nitrogen. Among these are *B. mesentericus* (which fixes appreciable quantities of nitrogen), *B. pneumoniæ*, *B. lactis viscusus*, *B. radiobacter*, *B. prodigiosus*, *B. asterosporus* and *B. amylobacter*.

Bredemann (20), after a careful study of the morphological and physiological characteristics of eleven "original species" of other investigators and of sixteen cultures prepared by himself from various soils, concluded that all belong to the single species *B. amylobacter* of Van Tiegham. Since this, however, there has been described at least one aerobic (168) clostridium. Moreover, Omelianskii (148) considers that the *Clostridium pasteurianum*, isolated from the Russian soils, is clearly a morphologically distinct race. An idea of the activity of some organisms in fixing nitrogen may be obtained from the following results reported by Löhnis (128). In every 100 cc. of 1 per cent mannite, or grape sugar soil extract, there was fixed, in the course of 3 weeks, nitrogen as follows:

	mgm.		mgm.
Microc. sulfursus	2.8 to 3.0	*Bact. chrysogloea*	1.4
Bact. prodigiosus	0.7 to 1.8	*Bact. tartaricus*	0.3
Bact. turcosus	0.3 to 1.6	*Bact. lipsiense*	0.2

C. B. Lipman (114) tested 18 organisms, including yeasts, pseudo-yeasts, and molds, nearly all of which showed a more or less pronounced power of fixing atmospheric nitrogen.

Pringsheim (159) has isolated from ordinary garden soil certain thermophilic organisms which fix from 3 to 6 mgm. of nitrogen per gram of dextrose when incubated at 61°C. in a Winogradsky's solution to which a little soil extract was added. Duggar and Davis (36) have recently investigated the subject of the fixation of nitrogen by the filamentous fungi, *Aspergillus niger, Macrosporium commune, Penicillium digitatum, Pexpansum, Glomerella, Gossypii,* and *Phoma betæ;* and of these only the last-named was definitely proved to be able to fix nitrogen. It is thus seen that the power of fixing nitrogen is a characteristic possessed by many microorganisms, in contradiction to the supposition of Winogradsky that this power is limited to a particular, or, at most, a few species. This is especially emphasized by the recent work of Emerson (39) who examined soil which contained 2,400,000 organisms which would develop on nitrogen-free media. Of these, 97 per cent possessed the power of fixing nitrogen; they constituted at least four distinct groups. Nevertheless, the most important group yet discovered is the *Azotobacter,* and it is with these mainly that this paper deals.

DISTRIBUTION

The nitrogen-fixing organisms are widely distributed, occurring in most soils. Lipman and Burgess (117), who studied the nitrogen-fixing flora, especially those of the *Azotobacter* group, of 46 soils from Egypt, India, Japan, China, Syria, the Hawaiian Islands, Guatemala, Costa Rica, Spain, Italy, Russia, Mexico, Asia Minor, Canada, Unalaska, Samoa, Australia, Tahiti, Belgium, Queensland, and the Galapagos Islands, found every soil possessed the power of fixing nitrogen in mannite solution. About one-third of the soils contained *Azotobacter;* frequently the same soil showed the presence of two or three different species of *Azotobacter. A. chroococcum,* however, was the most prominent. It was also found most widely distributed in the various soils. Groenewege (62) found *Azotobacter* in all but one of a series of Java soils.

Several hundred Utah soils have been examined and all found to fix nitrogen (55), many of them without the addition of carbohydrates. Aerobic *Azotobacter* are present in nearly all Utah soils. Hutchinson (85) found the *Azotobacter* in all the Indian soils examined. They occur in cultivated more frequently and in greater numbers than in virgin soils. This probably accounts for the much higher nitrogen-fixing power of cultivated soils.

Azotobacter were found in only two out of 64 localities in the soils of Danish forests (204). Both of the soils which gave positive tests were from beech-wood forests and contained calcium carbonates. Although the soils of these forests rarely contain enough carbonate to effervesce, they are usually neutral

or slightly alkaline. They contain calcium, but in forms other than the carbonate. It is generally understood that *Azotobacter* occur commonly in soils which contain sufficient calcium carbonate to effervesce when acid is added and that they scarcely ever occur in acid soils. Their disappearance from a soil is usually due to the absence of basic substances, especially of calcium and magnesium carbonate, and not to the presence of toxic substances (28). However, they are frequently not present in peaty soils, where their absence cannot be attributed to a lack of lime (41).

The aerobic nitrogen-fixers are probably more widely distributed in soils than are the anaerobic, for, although both groups are generally found in the Russian soils (148), the aerobic are found in the sands of Kirghese steppes and in the peat soils of the Province of Archangel in which the anaerobic forms are absent. Anaerobic nitrogen-fixers are, however, quite widely distributed in soils and are at times found on the leaves of forest trees (68).

The nitrogen-fixing organisms are confined almost entirely to the first three feet of soil (115), although they have been found in soil at all depths down to the tenth foot in the very favorable constituted loess soils of Nebraska (200).

They are most active in the upper few inches of soil, as is indicated by results obtained by Ashby (2).

SOIL	DEPTH	AVERAGE NITROGEN FIXED
	cm.	*mgm.*
Little Hoos	10	9.23
Little Hoos	20	7.29
Little Hoos	30	4.60

Reports on some Hawaiian soils (150) show them to be equally active at all depths to 4 feet, but this must be considered an exception, for the examination of numerous soils in Utah (57) has shown a gradual decrease in nitrogen-fixing powers with depth. The average of several hundred determinations, in both solution and soil media, are given below:

DEPTH OF SAMPLE	NITROGEN FIXED IN 100 GM. OF SOIL + 1.5 GM. OF MANNITE	NITROGEN FIXED IN 100 CC. OF ASHBY'S SOLUTION WITH 1.5 GM. OF MANNITE
	mgm.	*mgm.*
First foot	5.28	2.11
Second foot	2.42	0.77
Third foot	1.55	0.58

These samples were collected with such great care that there was no possibility of the mixing of one foot section with another. It is interesting to note that while the actual gain in nitrogen per gram of mannite is over twice as

great in the soil as in the solution, yet the relative gain per foot section is the same in both. There is about one-half as much nitrogen fixed in the second as in the first foot, and one-fourth as much in the third as in the first.

The nitrogen-fixing organisms are not confined to the soil alone, for Beijerinck and Van Delden first isolated *Azotobacter agilis* from canal water in Holland (9). *Azotobacter chroococcum* and *B. Clostridium pasteurianum* are both found in many fresh and salt waters (11), living on algæ and plankton organism (10).

REACTION OF THE MEDIA

The distribution and the physiological efficiency of the nitrogen-fixing organisms, especially of the *Azotobacter* species, are governed by the physical and chemical properties of the soil, foremost among which is the basicity of the soil, namely, its calcium or magnesium carbonate content (27). Ashby (1) bases his method for obtaining pure cultures of *Azotobacter* upon this property, for he finds that by picking out the crystals of the carbonate from the soil and seeding them into nitrogen-free media the likelihood of obtaining the organism is greatly increased. The addition of calcium carbonate to a soil often increases its azofying power (6), the extent of which increase depends on the lime requirements of the soil and on the fineness of the added limestone (102).

Christensen (28) has suggested that the *Azotobacter* be used as an index to the lime requirements of a soil. The test should include both a search for the organism in the soil and a test of their ability to grow when inoculated into the soil. He and Larson (29) examined more than one hundred soils of known lime requirement. They determined the carbon dioxide set free by acids, the amount of calcium dissolved by an ammonium chloride solution, the behavior of the soil toward litmus, and the biological test. The result of this test was that the biological test agreed with the known condition in 90 per cent of the cases, the ammonium chloride in 50 per cent, the litmus in 40 per cent, and the carbon dioxide failed more often than not to indicate the correct condition of the soil.

Fischer (43) failed to find *Azotobacter* in a heavy loam soil containing only 0.145 per cent of lime, while adjoining limed plots had an *Azotobacter* flora. The quantity of calcium carbonate which must be added to obtain maximum fixation varies with the soil (81).

A West Virginia Dekalb silt loam (6), which required 0.175 per cent of calcium carbonate to render it neutral by the Veitch method, gave greatest nitrogen fixation when 0.375 per cent of calcium carbonate was added. Above this concentration azofication decreased, but when phosphorus was applied with the lime it was not toxic even when present in quantities as great as 0.5 per cent. It is certain that large quantities of calcium carbonate may be present in soil without injury to the azofiers (139).

The author found numerous *Azotobacter* and a very active nitrogen-fixation in a soil 43 per cent of which was calcium and magnesium carbonate (59).

The organisms develop normally in the presence of either calcium or magnesium carbonate, but in liquid cultures the film develops earlier and it contains less foreign organism in the presence of magnesium carbonate than in the presence of calcium carbonate. The actual nitrogen fixed, as reported by Ashby (1), is also greater where the magnesium carbonate is used. This he attributes to the suppression by the magnesium of foreign organisms, especially of the butyric acid ferments.

There is, however, a marked difference in the action of calcium carbonate and magnesium carbonate when they are applied in large quantities. Lipman and Burgess (116) found the calcium carbonate stimulating and never toxic to *Azotobacter chroococcum* in concentrations up to 2 per cent in mannite solution. The magnesium carbonate was sharply toxic in higher concentrations above 0.1 to 0.2 per cent in such cultures. The calcium salt is without effect when added to most soils up to 1.4 per cent, but the magnesium carbonate is even more toxic in soils than in solutions. Moreover, their work indicates that calcium exerts a protective influence, in both soils and solutions, against the toxic influence of magnesium. The best ratio of calcium to magnesium varies with solution and soil.

In many soils lime increases the nitrogen fixed, for Krzemineniewski (113) found limed soil to fix in 10 days 17.52 mgm. of nitrogen, whereas adjoining unlimed soil fixed only 7.15 mgm. There is, however, the possibility of applying too large a quantity of the caustic lime and thereby decreasing nitrogen-fixation (95), a condition which has never been experienced in the use of the carbonate.

Von Feilitzen (41), however, found neither a direct relationship between lime content of moor soil and the development of *Azotobacter*, nor relationship between their development and the reaction of the soil. But this only serves to illustrate the fact that although lime and neutral or slightly alkaline media are essential, they will not insure a rich *Azotobacter* flora in a soil unless all other conditions are optimum. Remy (165) found sodium and potassium carbonate less favorable for nitrogen-fixation than were calcium or magnesium.

So far as the writer is aware, Krainskii (105) is the only worker who has found sodium carbonate more favorable than calcium carbonate. This may have been due to the sodium carbonate's liberating plant-food which was in the soil in an insoluble form but which was essential to the development of *Azotobacter*. Mockeridge (139) has found that the presence of sodium salts is unnecessary and depressing at least to the growth of *Azotobacter*. The beneficial effect ascribed to sodium chloride solution in inoculating agar plates is due to the fact that this liquid is isotonic with the cell content solution, but the sodium hydroxide is a far less advantageous neutralizing agent than is calcium or magnesium carbonate (139). Furthermore, Lipman failed to stimulate the azofiers with any of the sodium salts.

FOOD REQUIREMENTS OF THE AZOFIERS

These organisms probably require for their nutrition the same elements as do the higher plants, namely carbon, hydrogen, oxygen, nitrogen, potassium, phosphorus, sulfur, calcium, magnesium, and iron, and possibly aluminum and manganese.

They obtain their carbon and hydrogen from organic compounds, preferably from carbohydrates, which are considered in detail under sources of energy. Oxygen is obtained either from the atmosphere or from combined sources depending on the species and the conditions under which they are grown.

A marked difference between these and the higher plants is that they possess the power of obtaining their nitrogen from the air, but in the presence of combined nitrogen they obtain but little from the air (191). Lipman (122), Stranak (191), Heinze (74), and Stoklasa (187) found that small quantities of nitrates stimulated *Azotobacter*, whereas large quantities discouraged nitrogen-fixation since the organisms live on the nitrates. This is the case whether the nitrates are added to the soil or to the solution in which nitrogen-fixation is taking place. Coleman (30) considers this action as due to several different factors: namely, (a) a direct toxic action of the salt, (b) antagonism of other organisms which it favors, (c) the using up of the energy supply by these organisms, and (d) the discouragement of fixation by the use of sodium nitrate. The last would seem to be the most important factor when viewed in connection with the following results reported by Hills (77):

TREATMENT NITRATE	RELATIVE NUMBER OF ORGANISMS			RELATIVE PER CENT OF NITROGEN FIXED			
				Sterilized soil		Unsterilized soil	
	KNO_3	$NaNO_3$	$Ca(NO_3)_2$	$NaNO_3$	$Ca(NO_3)_2$	$NaNO_3$	$Ca(NO_3)_2$
mgm.							
0	100	100	100	100	100	100	100
10	348	191	362	100	105	240	219
50	8,210	3,150	4,528	342	371	500	444
150	12	117	763				
200	0	0	0	352	467	879	557

The number of organisms developing and the nitrogen fixed in the one receiving no nitrate is taken as 100 per cent.

It is quite evident from these results that although nitrates cause more active multiplications of *Azotobacter*, it greatly reduces their physiological efficiency. The organisms used by Hills had probably grown for a long time on media poor in nitrogen, and their ability to fix nitrogen was, therefore, high. But would they continue to exert this power if grown on media rich in nitrogen? The evidence points strongly to the conclusion that they

would not. It is certain, however, that the nitrates are toxic in comparatively low concentrations. Nitrates and ammonium sulfate are rather effective in stimulating nitrogen-fixation when the *Azotobacter* are grown in connection with the cellulose ferments (136). Even here, however, large quantities decrease this power. In pure cultures ammonium sulfate (108, 122) seriously retards nitrogen-fixation, whereas the nitrogen of humus, even in large quantities, appears to have no serious retarding influence (65). Nevertheless, a high nitrogen content of soils seems to be unfavorable to vigorous nitrogen-fixation (117). Whether this would be the case where the nitrate content of the soils is kept low but with the readily-decomposable protein nitrogen high is yet to be answered. Hiltner and Störmer (79) consider that when the nitrogen content of the soil passes beyond a certain limit, the decay bacteria increase rapidly, and in the struggle for existence they are able, with the advantage at their disposal, to suppress the more slowly growing *Azotobacter*.

Potassium is essential to the higher plants and cannot be replaced entirely by related substances, yet Gerlach and Vogel (50, 51, 52) early reached the conclusion that potassium and magnesium are not essential to the *Azotobacter*. Their results were, however, generally considered erroneous, for while as much nitrogen was fixed in 20 days without as with potassium, after 40 days there was no further fixation in the solution without potassium, but in its presence the nitrogen gain nearly doubled. It was, therefore, argued that the traces of potassium left in the chemicals and dissolved from the glass during sterilization had been enough to permit development for a time. If these elements are essential, it must be in extremely minute quantities, for Vogel (197), using the purest chemicals obtainable, was able to prepare potassium-free media in which the *Azotobacter* developed. He did find, however, that potassium favors their development.

Phosphorus is required by these organisms (72, 206), large quantities being used for the building of the nucleo-proteins and phospho-proteins in which their bodies are extremely rich. Moreover, it greatly accelerates the reaction and economizes the carbohydrates; hence it is rather evident that phosphorus plays a very essential part in *Azotobacter* metabolism. Possibly in the early stages of the process a definite chemical reaction occurs between the phosphate and the carbohydrate similar to that occurring in alcoholic fermentation (67).

$$\text{I.}\quad 2C_6H_{12}O_6 + 2R_2HPO_4 \rightarrow 2CO_2 + 2C_2H_2O + C_6H_{10}O_4(PO_4R_2)_2$$
$$\text{II.}\quad C_6H_{10}O_4(O_4R_2)_2 + 2H_2O \rightarrow C_6H_{12}O_6 + 2R_2HPO_4$$

The *Azotobacter* are able (199) to utilize the phosphorus of di- and tri-basic sodium and potassium phosphate and of dibasic calcium phosphate (98). Mockeridge (139) obtained an increase of 23 per cent in nitrogen-fixation with basic slag. There were two maxima, one with 0.4 per cent, the other with 1.0 per cent slag. This is attributed to the stimulating effect of the iron and

manganese in the slag, the maximum effect of one being produced at 0.4 per cent, the other at 1.0 per cent. The tribasic calcium phosphate, bone ash, iron and aluminum phosphate all serve only as difficultly available sources of phosphorus. Raw rock phosphate and bone meal fail entirely to furnish enough available phosphorus for the development of *Azotobacter* (27).

The addition of phosphorus to a soil often greatly increases azofication (6).

TREATMENT	WITHOUT P	WITH P
	mgm.	*mgm.*
No lime	0.6	0.9
Lime	1.5	4.6

Moreover, Christensen (27) has found soils in which phosphorus is the limiting element in *Azotobacter* growth. He entertains the hope that in view of the relationship between *Azotobacter* growth and lime and phosphorus that it will become eventually possible by the determination of bacterial food requirements to secure a general expression for the soil content of plant-food available to crops. He (28) further suggests that where a mannitol solution free from phosphorus produces a vigorous growth of *Azotobacter* after inoculation with a soil, it may be assumed that the soil is not deficient in available phosphorus. Dzierzbicki (38) notes that if soils are deficient in available lime, phosphoric acid, or potash, nitrogen-fixing bacteria such as *Azotobacter* are either entirely absent or present only in small numbers.

There is a definite relationship between the carbon and phosphorus content of a soil and the nitrogen assimilated. According to Stoklasa (189) *Azotobacter* assimilates from 5.0 to 5.7 gm. of free nitrogen for every gram of phosphorus used. Although these organisms are directly dependent upon a readily-available supply of phosphorus to promote growth, they do not change it into the organic form as rapidly as do the ammonifying bacteria.

Sulfur is required by the azofiers possibly for the formation of the proteinaceous material of their bodies. It is certain that the benefit derived by *Azotobacter* from the sulfates of iron and calcium is due in a large measure to the sulfur which these compounds supply. No evidence has as yet been produced which would lead us to believe that the organisms can use sulfur as a source of energy.

Calcium carbonate and calcium oxide, in addition to furnishing a base which neutralizes the acid formed in the metabolic processes of the *Azotobacter*, also furnish calcium to the organism. Christensen (27) brought out the fact that *Azotobacter* can derive their calcium from dibasic calcium phosphate and some calcium salts of organic acids. They could not, however, utilize the calcium of tribasic phosphate, of calcium chloride or sulfate.

Iron (95) is essential and either the ferric or ferrous sulfate is especially beneficial (98). Rosing (169) found the amount of nitrogen fixed increased

from 2.23 mgm. to 10.3 mgm. per gram of mannite when iron sulfate was added to the cultural media. This is due, in a great degree, to the iron which serves as food for the organism, yet its colloidal nature may play a part, for both organic and inorganic colloidal substances have an especially favorable action on *Azotobacter*, although the action of the inorganic colloids is fully manifest only in the presence of organic colloids (155). If used alone, large quantities of the ferric hydroxide are essential for the maximum effect, but in the presence of organic colloids, very small quantities of iron are effective. This has been attributed to the action of the colloidal iron which absorbs the nitrogen and oxygen of the air and brings them into more intimate contact with the *Azotobacter* (178). This would not only accelerate the normal processes of the aerobic *Azotobacter* by furnishing them with nitrogen and oxygen but it would tend to suppress the anaerobic processes which are extremely wasteful of the food. According to Kaserar (88), these organisms also require aluminum. Although this may accelerate, it has not been proved to be essential to their growth.

While not essential to the organisms, manganese is an extremely active catalyzer (61) in increasing proportions up to 6 mgm. per 100 cc. of media. Above this concentration the reaction falls off rapidly, and at 20 mgm. it is less than in the absence of manganese. It is oxidized by *Azotobacter*, and in the proportion of 1 part to 200,000 parts of soil it is an active stimulant. Olaru (146) considers it likely that the increased yield obtained after the application of manganese compounds to a soil is due to its accelerating the action of the nitrogen-fixing organisms of the soil.

ALKALI SALTS

In addition to the essential elements of plant-food applied to a soil, other so-called soil amendments are often added. These may influence the physical, chemical, or bacterial properties of the soil. Some substances may alter the physical properties of the soil to such an extent that the bacterial flora is modified. Others may react chemically with constituents within the soil and in so doing liberate substances which can be utilized by the bacteria. Again, there may be a direct stimulation or retarding effect upon the organisms. Within this field there is much yet to be learned concerning the nitrogen-fixing organisms. We have, however, some information concerning the influence of the so-called alkalies upon the nitrogen-fixing organisms. A large number of analyses have shown that sodium salts are not necessary for the activity of the *Azotobacter* (139), nor are they stimulated by the common soil "alkalies—sodium chloride, sodium sulfate or sodium carbonate" (118). In this latter respect they differ greatly from the ammonifying and nitrifying organisms.

They are, however, quite resistant to these compounds, as may be seen from the following reported by Barnes and Ali (4).

Nitrogen fixed per gm. of mannite in nutritive solution inoculated with salt land..... 1.23
Nitrogen fixed per gm. of mannite in nutritive solution inoculated with sterile soil..... 7.80
Nitrogen fixed per gm. of mannite in nutritive solution inoculated with normal soil..... 7.07

Soil which contained sufficient salt to check all vegetation contained nitrogen-fixing organisms. Barnes and Ali hold that salts do not accumulate in the soil in sufficient quantities to kill the nitrogen-fixing organisms, but they are rendered inactive and as soon as the salts are leached from the soil the *Azotobacter* commences to work. Keutner (92) who worked with marine forms of the azofiers, found they would grow and assimilate nitrogen in an 8 per cent solution of sodium chloride. Nitrogen-fixers growing in arable soil would not be as resistant as are those which have become adapted to a medium with a high osmotic pressure, but *Azotobacter* in general appear to be more resistant to alkali salts than are most other soil organisms, for no toxic influence was noted by Lipman (118) until the concentration of sodium chloride in the soil reached 0.5 per cent, sodium sulfate 1.25 per cent, and sodium carbonate 0.4 per cent. They are much more sensitive to sodium in the form of nitrates, for 0.15 per cent stopped their multiplication and probably killed many of them (77).

NON-VOLATILE ANTISEPTICS

Arsenic, lead, copper, etc., when applied to soil in the form of lead arsenate, sodium arsenate, arsenic trisulfid, or zinc arsenite, stimulate azofication (56). This is greatest with lead arsenate and least with zinc arsenite. Paris green not only does not stimulate, but is toxic when the concentration reaches 120 parts per million. The toxicity, however, is due to the copper and not to the arsenic contained in it. Sodium arsenate becomes toxic when a concentration of 40 parts per million of arsenic is added, and when 250 parts per million are added it entirely stops nitrogen fixation. Lead arsenate is not toxic even at a concentration of 400 parts per million of arsenic. The toxicity of arsenic trisulfid and zinc arsenite is only slight at this concentration.

The stimulation occurring when arsenic is added to a soil is not due to any inherent peculiarity of one soil, for soils which differ greatly in physical and chemical properties have their nitrogen-fixing powers greatly increased when arsenic is applied to them. At least some soils high in organic matter fix as much nitrogen in the presence of arsenic and in the absence of mannite as they do in the presence of mannite and in the absence of arsenic. The stimulation is greatest when the water-soluble arsenic content of the soil is about 10 parts per million.

One type of *Azotobacter* has been isolated which is stimulated by arsenic, and in this case the stimulation is due to the organism utilizing more economically in the presence of arsenic its source of carbon than it does in the ab-

sence of arsenic. The arsenic compounds do not act as a source of energy to the organisms. The main part of the stimulation noted in the soil with its mixed flora is undoubtedly due to the arsenic inhibiting injurious species.

Arsenic cannot replace phosphorus in the vital process of the nitrogen-fixing organisms, but it may in some manner liberate the phosphorus from its insoluble compounds. This may be either a direct or an indirect action. Arsenic stimulates the cellulose ferments, which, in turn, react upon the activity of the nitrogen-fixing organisms. The nitrogen-fixing powers of soil extract, of filtered soil extract, and of soil dried for some time are only slightly stimulated by arsenic, showing that arsenic acts mainly by the removal of a thermolabile body which occurs in the soil.

In the experiments where lead has been applied to a soil it has been associated with arsenic, but the evidence that it stimulates nitrogen-fixing organisms when applied in small quantities is conclusive. The point at which it would become toxic is, however, unknown. Copper, on the other hand, is toxic even in the lowest concentration which has been so far tested.

VOLATILE ANTISEPTICS

Ether, carbon bisulfid, and other volatile (46) antiseptics usually (95, 98) increase nitrogen-fixation when added to the soil in small quantities. It is certain that they stimulate *Azotobacter* in pure cultures, but not to so great a degree as in mixed cultures. *A. chroococcum* is fairly resistant to carbon bisulfid (135), as it is killed in 24 hours in a solution containing 1.7 parts in 1,000 at 20°C., but it survives for 48 hours in moist soils which have been impregnated with the fumes of carbon bisulfide. Various theories have been advanced to account for these phenomena. These have been carefully analyzed by Kopeloff (103) and co-workers and will not be considered here in detail. Suffice it to state that the evidence points strongly to the conclusion that there are a number of factors at work, chief among which are the following:

(a) In small quantities the compounds directly stimulate the protoplasm of the organism and thus increase their physiological efficiency.

(b) Antiseptics simplify the bacterial flora of the soil, and the nitrogen-fixing organisms, being more resistant to the compounds than are some other organisms, survive and later multiply unhindered by other forms.

(c) The compounds may stimulate other organisms or classes of organisms which render the carbonaceous material of the soil more available to the nitrogen fixers, or possibly remove products which are injurious to them.

(d) The compounds may render more plant-food available in the soil. This may either be a direct interchange between the compounds and those of the soil or a dissolving of various substances surrounding the essential constituent, in either case liberating more available food for the *Azotobacter*.

It is interesting in this connection to note the explanation which Kruger and Heinze (109) make for the action of some forms of green manure on bac-

terial activity. It had long been known that mustard, when plowed under on nitrogen-poor soil, increased the next crop grown on that soil. It would appear from some facts already known that green mustard substances in the soil retard the formation of acid-forming species (72), thereby greatly simplifying the bacterial flora. These compounds seem to act upon the bacterial flora in a manner similar to that of a carbon bisulfid. These workers find theoretical support for this belief in the fact that allyl mustard oil, $C_3H_5 - N = C = S$, a constituent of the mustard plant, may be regarded as a derivative of carbon bisulfide.

ORGANIC SOIL CONSTITUENTS

Reed (164) found urea, glycocol, formamide, and allantoin active in depressing nitrogen-fixation. This he attributes to the compounds furnishing to the *Azotobacter* an available source of combined nitrogen and not to the direct toxic action of the compound. But Walton (201) found that the addition of urea, peptone, acetamid, asparagin, and casein to culture media had only a slight influence on the fixation of nitrogen by *Azotobacter*.

Caffeine, alloxan, betaine, trimethylamine, legumin, cinnamic acid, aspartic acid, asparagine, hippuric acid, creatin, creatinine, xanthine, and hypoxanthine, are all toxic to *Azotobacter* even in small quantities. Only the first two have been tested in concentrations dilute enough to stimulate, which is remarkable, as many of these compounds stimulate the higher plants and some can be utilized directly by the plant.

Esculin, vanillin, daphnetin, cumarin, pyrocatechin, heliotropin, arbutin, resorcin, pyrogallol, phloroglucine, hydroquinone, salicylic aldehyde, oxalic acid, quinic acid, dihydrostearic acid, rhamnose and borneol, on the other hand, do not stimulate in any concentration. Nor are they toxic until fairly large quantities have been added. In this regard the nitrogen-fixing organisms appear to differ greatly from the nitrifying bacteria and higher plants. The resistance of the nitrogen-fixers to various chemicals has likewise been called to our attention by Lipman (118) in his study of the influence of alkalies on nitrogen-fixation.

INFLUENCE OF COLLOIDS

It was recognized early in the study of nitrogen-fixation that when sterilized soil is added to a nutritive medium it greatly increased the quantity of nitrogen fixed. This condition is due to several factors and is partly explained by Krzemieniewski's (111) results wherein he found that nitrogen-fixation is decidedly increased by the addition of soil humus, either as free humic acid or as salts of potassium, sodium or calcium. Kaserer (88) maintains that this is due to the inorganic nutrients, especially to aluminum and silicic acid supplied to the microorganisms through the humus. This is probably true

in part, for the fixation varies with the humus derived from different sources. Moreover, artificial humus, prepared by boiling sugar with acids, fails to stimulate.

That much of the beneficial effect is due to the constituents in the humus appears likely from the results obtained by Sohngen (178) who found that colloidal iron oxide, aluminum oxide, and silicon oxide all greatly stimulated the nitrogen-fixing powers of *Azotobacter chroococcum*. This he attributed to the absorption of oxygen and nitrogen by the colloid, which he maintains would make them more readily available to the organism. The boiling of natural humus with hydrochloric acid would either remove the foreign material or change it from the colloidal form, and thus, as has been found to be the case, render it inert. Löhnis and Green (130) take exception to this explanation, for they found no absorptive action exerted by humus on either the nitrogen

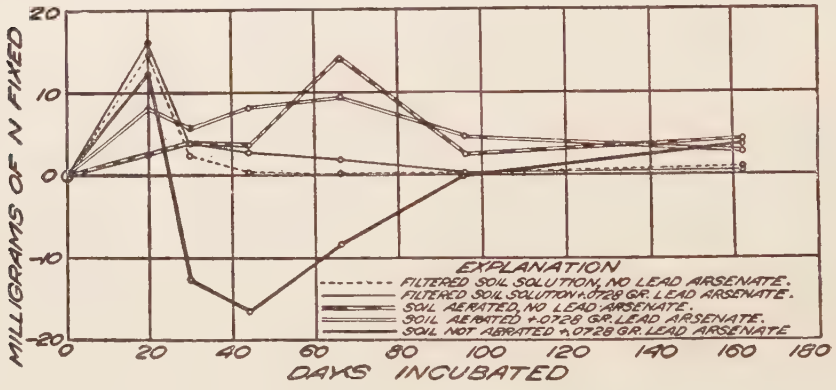


FIG. 1. GRAPH SHOWING THE EFFECT OF AERATION ON THE NITROGEN-FIXING ACTIVITY OF SOIL CONTAINING COMPOUNDS OF ARSENIC

or the oxygen. Furthermore, Rosing (169) found that he could stimulate just as effectively with iron as with humic acids. But much larger quantities of colloidal iron are required when it is used singly than when used in conjunction with an organic colloid (155). The extent of the stimulation resulting varies with the form in which the iron is applied and is most effective in the form of the hydroxide and in the presence of cane sugar (166). In this case it is probably the saccharate which is the active substance. Hence, the contradictory results reported may be due to the different mineral constituents of the humus.

These facts make it certain that colloids of the metals act as stimulants to nitrogen-fixing bacteria, as does also crude humus (141). Carefully purified humates do not possess this property, but it is possessed by the aqueous extract, the alcoholic extract, and the phosphotungstic fraction of the aqueous extract from "bacterized" peat. Whether this influence is due to a catalytic

effect, as suggested by Sohngen, or whether the substance furnished a direct source of nutritive material is not clear at the present time.

Moreover, the colloid may act as a protection to the organism against poison (56); for, when 10 parts per million of soluble arsenic is maintained in a soil, it acts as a stimulant to *Azotobacter*. If, however, this proportion is added to the Ashby nutritive solution it stops all nitrogen-fixation. This is due in part to the absorption of the arsenic by the soil. This absorption would have to be attributed largely to the silica compounds, for the nitrogen-fixing organisms are stimulated by arsenic in quartz free from organic colloids. This could readily be due to the arsenic becoming concentrated at the surface layers of the silica, leaving the inner part of the water film comparatively free from arsenic, in which part of the water film the microorganisms multiply and carry on their metabolic processes. This being the case, one should and probably could find a water solution weak enough to stimulate bacteria. A great difference, however, between the solution and the sand-culture method is the greater aeration in the sand. That the aeration of a culture medium does play an important part in determining the activity of the nitrogen-fixing powers of a soil is strikingly brought out in figure 1.

SOURCES OF ENERGY FOR THE AZOTOBACTER

The nitrogen-fixing organisms differ widely from other plants in their energy requirements. This is due to the fact that they are carrying on endothermic reactions in which nitrogen is concerned. This necessitates a greater supply of energy than is required by other bacteria. They are similar to most other bacteria in that this energy must be supplied by an organic compound, preferably one of the carbohydrates.

Berthelot (12) in his early work maintained that the gains in nitrogen noted in some soils were due to the action of biological agents on the humus of the soil. This was followed by the observation by others (75, 192, 84) that when forest leaves are allowed to decompose in soil there is an increase in its nitrogen content. Koch (98) in 1907 increased nitrogen-fixation by the addition to soil of dextrose, cane sugar or starch, but there was practically no increase when straw, filter paper or buckwheat was applied. Yet Stoklasa (187) showed that the decomposition products of these substances acted as a valuable source of energy to the *Azotobacter*, and Stranak (191) considered that the pentosans of the soil are of the greatest importance in the assimilation of nitrogen by soil bacteria.

A fair idea of the great variety and relative efficiency of substances which may serve as a source of energy to the azofiers may be obtained from the work of Löhnis and Pillai (132). They inoculated a nutritive solution with 10 gm. of soil and after 10 days determined the gain in nitrogen.

SUBSTANCE ADDED	NITROGEN FIXED AFTER 10 DAYS	SUBSTANCE ADDED	NITROGEN FIXED AFTER 10 DAYS
	mgm.		*mgm.*
Mannite	9.40	Starch	3.36
Xylose	9.54	Sodium tartrate	2.82
Lactose	9.12	Glycerine	1.68
Laevulose	8.52	Sodium succinate	2.96
Inulin	7.72	Calcium lactate	2.49
Galactose	7.86	Sodium citrate	1.42
Maltose	7.44	Sodium propronate	1.10
Arabinose	7.62	Potassium oxalate	0.12
Dextrin	7.18	Calcium butyrate	0.02
Sucrose	8.60	Humus	−0.96
Dextrose	4.62		

Other workers have noted larger gains of nitrogen than those noted by Löhnis and Pillai, but they can readily be attributed to (a) the time of incubation (81)—in this case, 10 days being far too short for the complete utilization of the carbonaceous substance applied; (b) the species of nitrogen-fixers which are bringing about the change; and (c) whether pure or mixed cultures are used. The order of effectiveness noted above, however, is that recognized by most workers. Brown and Allison (23), however, do report results in which greater fixation was obtained with dextrose than with mannite. But in this case, calcium or sodium carbonate seems to be even more necessary than it is with the mannite (186). Moreover, some species utilize one carbohydrate most effectively and another species a different one. To this list may be added malate, gum tragacanth, ethylene glycol, methyl, ethyl, and propyl alcohols, lactic, malic, succinic and glycollic acids. Fatty acids are readily utilized, the amount of nitrogen fixed being greater with the increased molecular weight, from 1.47 mgm. with formic acid, to 6.08 mgm. with butyric acid (140). Most of the naturally-occurring glucosides and many benzine derivatives are unsuitable as sources of energy for *Azotobacter*. Molasses, which should serve as a useful source of energy (95), often results in a loss of nitrogen when applied to the soil. This may be due to the time of applying, concerning which Peck (150) maintains that molasses applied to a land lying fallow at an interval of several weeks before planting of the crop may produce beneficial results by increasing nitrogen-fixation.

Beijerinck early recognized that certain decomposition products of cellulose can also serve as sources of energy for *Azotobacter*, and Pringsheim (157) found that *Clostridium americanum* does not fix atmospheric nitrogen in sterilized cellulose unless other carbohydrates like dextrose, lactose, mannitol, or sucrose are present. However, in the presence of cellulose, *Clostridium* will fix nitrogen and this more efficiently than it will in the regular carbohydrate medium. The same holds for agar (161). Just how completely cellu-

lose must be broken down before it can be utilized by *Azotobacter* is not definitely known, but it is known that *Azotobacter* cannot utilize cellobiose except when grown in conjunction with *Aspergillus niger*. It is, therefore, certain that the products which are utilized by the *Azotobacter* are comparatively simple.

Cellulose when applied to the soil may serve as a valuable source of energy, provided sufficient time is allowed for its decomposition (17, 97, 158). The cellulose ferment is probably the most efficient organism in the soil in bringing about this decomposition (136). But the number of soil fungi which possess this power are numerous (202).

Hoppe Zeyler (83) thinks that cellulose is decomposed according to the following formula: (a) the hydration of the cellulose with the formation of hexose, $C_6H_{10}O_5 + H_2O = C_6H_{12}O_6$; (b) the destruction of the carbohydrate with the formation of equal quantities of carbon dioxide and methane, $C_6H_{12}O_6 \rightarrow 3CO_2 + 3CH_4$. None of the cellulose ferments studied by McBeth (137), however, yielded gaseous products on cellulose or sugar; hence the *Azotobacter* probably gets from the cellulose ferments, pentoses, hexoses, or slightly simpler products upon which they could readily fix nitrogen.

At times in fermenting straw and manure, the thermophilic anaerobic bacteria play a major part, in which case fatty acids probably make up the greater part of the end products (160).

It is claimed by Dvarak (37) that substances with low carbon and high oxygen content are usually the best sources of energy for *A. chroococcum*, which assimilated 5.73 mgm. of free nitrogen per 100 gm. of carbon in pine leaves as compared with 1237.9 mgm. per 100 gm. of carbon in red clover. He obtained for other substances the following results:

1456.5 mgm. of nitrogen per 100 gm. as glucose.
280.4 mgm. of nitrogen per 100 gm. as corn stalks.
596.8 mgm. of nitrogen per 100 gm. in stalks and root residues of corn.
325.4 mgm. of nitrogen per 100 gm. in wheat straw.

The carbon-nitrogen ratio (23) in compounds is no indication of their value to nitrogen-fixing organisms, for non-leguminous hays and straws are utilized just as effectively as are the legumes. Mockeridge (140) found that the ratios of nitrogen fixed to the heat of combustion with the four lower fatty acids is almost constant. The same holds true with starch, dextrin and gum arabic, when allowance is made for experimental error, which is greater with these compounds than with the simpler compounds. This close relationship is not, however, general and no such graduated uniformity is observed with the series of monohydric alcohols.

The quantity of nitrogen fixed per gram of carbohydrate varies greatly with the species. Winogradski (210) found *Clostridium pasteurianum* to

assimilate 2 to 3 mgm. of nitrogen for each gram of sugar. But this, like other anaerobic organisms, is very wasteful of energy, leaving much of it in the butyric acid, acetic acid, and butyl alcohol formed. In the experiments of Bredeman with *B. amylobacter* and of Pringsheim with *Clostridium americanum*, the amounts fixed were at times much larger. Much greater fixations have been reported with *Azotobacter*, and Lipman has obtained as high as 15 to 20 mgm. of nitrogen per gram of mannite assimilated by *A. vinelandii*. This quantity is considerably greater than that fixed by any of the other members of the group.

Koch and Seydel (100) claim that the usual method of estimating the nitrogen-fixing powers of *Azotobacter* is erroneous, as it does not represent accurately the intensity of the process. In a series of experiments made by them, the amounts of nitrogen fixed per gram of dextrose used were 53, 70 to 80, 20 to 30, and 5 to 8 mgm. on the first, second, third, seventh, and eighth days, respectively.

Krainskii (107) considers that there should be sufficient organic matter in the soil to permit that for 1 part of nitrogen formed there will be 90 parts of carbon for the use of the organism. The organisms, however, utilize the carbohydrates more economically when only small quantities are present (81). Walton (201) finds with Ind an soil that highest fixation is obtained per gram of mannite when 10 gm. are used in 1 litre of nutritive solution. Young, vigorously-growing cultures usually fix more nitrogen than older ones (52). The nitrogen fixed is greatest in the first stages of the growth of the organisms, as is seen from figure 2 from the work of Omelianski (148).

The efficiency of these organisms is, therefore, greatest when they are rapidly multiplying and it decreases as their metabolic products accumulate (139). Hoffman and Hammer (81) claim this to be due in impure cultures to a loss of nitrogen or free ammonia occasioned by the decomposition of the cells of *Azotobacter*. This explanation would hardly hold in the presence of pure cultures, unless we ascribe the breaking down to an autolytic ferment secreted by the *Azotobacter* cell. According to Koch and Seydels (100) this indicates that in the latter stages of fixation, when there occurs an accumulation of nitrogenous material in the medium, the organisms employ the carbohydrates for other purposes than for nitrogen-fixation. Under natural conditions in the soil this accumulation and concentration of nitrogenous material by the *Azotobacter* is not likely to occur; hence, they assume that the organism will continue fixing nitrogen at the high ratio noted in the early part of laboratory experiments.

The quantity of nitrogen fixed, however, is dependent upon factors other than the source of energy; e.g., Krzemeniewski (112) found in experiments with *A. chroococcum* that the addition of humates to the cultural solutions increased the nitrogen fixed from a maximum of 2.4 mgm. to 14.9 mgm. Moreover, Krainskii (106) found *Azotobacter* to utilize from 100 to 200 gm. of

sugar in the assimilation of 1 gm. of nitrogen when grown in solution, but when grown on sand it required only 11 to 30 gm. for the same fixation.

They utilize their energy more economically in the presence of a liberal supply of phosphorus than when the quantity of available phosphorus is limited (38). This accounts, in a measure, for the high fixation noted in most Utah soils.

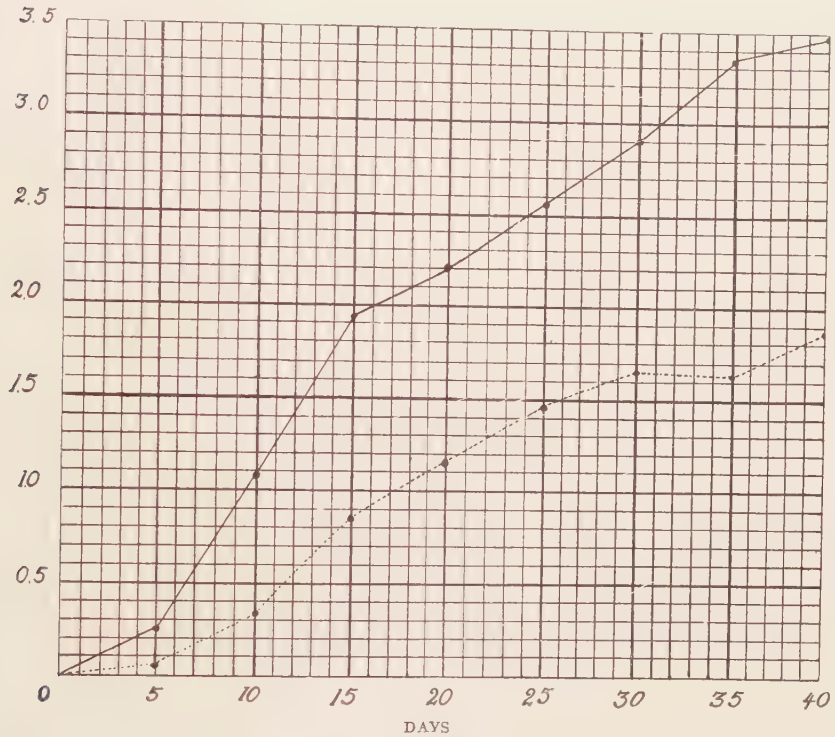


FIG. 2. GRAPH SHOWING THE FIXATION OF NITROGEN AND DECOMPOSITION OF SUGAR IN MIXED CULTURES OF *Azotobacter chroococcum* AND *Clostridium pasteurianum*

MANURE

It has been known for a long time that humus exerts a highly favorable effect on nitrogen-fixation. The great question, however, has been as to the manner of action. Humus, being such a complex variable substance, varies greatly in action, depending upon its source (111). Remy (165) considered that some of the products from humus are favorable sources of organic matter for *Azotobacter*. Definite and valuable information is furnished by the work of Löhnis and Green (130). They worked with mixed cultures of *A. chroococcum*, *A. beijernickii*, *A. vinelandii*, and *A. vitrium* in Beijernick's mannite solution with various forms of organic matter.

MATERIAL	NITROGEN FIXED IN 100 CC. OF SOLUTION AFTER 3 WEEKS
	mgm.
Fresh straw	10.0
Fresh stable manure	9.8
Fresh peat	9.3
Green manure	8.0
Beijerinck's mannite solution	5.6

After humification, these substances were even more readily assimilated and the nitrogen-fixation was greater than when the unhumified substance was used.

The same year Hanzawa (65) published results which show that stable manure even up to 3 per cent greatly stimulated the bacteria' activities. Green-manure humus was found by him to be injurious. From this it is certain that humus can act as a source of energy and usually stimulates bacteria, but the extent is governed largely by its composition and by the quantity of available combined nitrogen which is being supplied with it to the organism. In addition to this, corn roots (37), corn stalks, oak leaves, lupine alfalfa, maple leaves, and pine needles may all serve as a useful source of energy to the nitrogen-fixing organisms. Apparently, the tissues from the non-legume give a greater gain than do those from the legumes (23). Fulmer (48) has recently confirmed these results.

The influence of stable manure upon the nitrogen-fixing powers of the soil under field conditions is seen from the following table in which the quantity of nitrogen fixed in the unmanured soil has been taken as 100 per cent (58).

TREATMENT	NITROGEN FIXED
	per cent
No manure	100
5 tons of manure per acre	103
10 tons of manure per acre	110
15 tons of manure per acre	105
20 tons of manure per acre	103
25 tons of manure per acre	101

These results indicate clearly that stable manure has a beneficial effect upon the nitrogen-fixing powers of the soil, but if used in large quantities the benefit is not so pronounced as if used in smaller quantities.

This decrease in nitrogen-fixation with increased additions of manure must be considered as due to its physical effect upon the soil, for Richards (167) found that *Azotobacter* grow and fix nitrogen in horse manure when it is well aerated and contains sufficient moisture and calcium carbonate. There is, too, a close connection between the diet and the effect. Horses fed on oats

gave feces which induced the greatest fixation; horses on grass next; cattle receiving oatmeal cake third; but the feces from cattle fed on grass proved unsuitable.

Manures often contain nitrogen-fixing organisms of considerable activity. Their activity appears to be greatest in fermenting manures mixed with straw which serves as a source of energy (194).

Although Fulmer and Fred (49) were unable to find *Azotobacter* in any of the samples of manure examined, they did obtain many nitrogen-fixing bacteria from it. One of these organisms, for which they suggested the name of *B. azophile*, is as efficient in fixing nitrogen as is *Azotobacter*. This would make it appear that manure may often carry to the soil nitrogen-fixing organisms.

METABOLISM OF AZOTOBACTER

Much time has been given to a study of the metabolism of *Azotobacter*, yet our knowledge of this subject is far from satisfactory. It is well known that the organisms oxidize the various carbohydrates and with the energy thus obtained build up complex nitrogen compounds. Berthelot (13) early recognized that the nitrogen so fixed is insoluble in water, thus indicating its protein nature. Lipman (123) found that there was a small but appreciable quantity of nitrogen in both young and old cultures of *A. vinelandii* not precipitated by lead acetate and a large proportion not precipitated by phosphotungstic or by tannic acid. Further work indicated that the substance was either amino-acids or comparatively simple peptids. He considered that one of early substances synthesized by these organisms is alanin. An analysis of the *Azotobacter* membrane gave the following results:

NITROGEN AS AMMONIA	BASIC NITROGEN	NON-BASIC NITROGEN	NITROGEN IN MgO PRECIPITATE	TOTAL PER CENT OF NITROGEN
per cent	*per cent*	*per cent*	*per cent*	*per cent*
0.98	2.76	6.39	0.42	10.45

This he finds corresponds remarkably close with that of legumin. Experiments with plants indicate that the nitrogen of the *Azotobacter* cells is not readily assimilated by plants (72).

Stoklasa found the *Azotobacter* cells to contain 10.2 per cent of total nitrogen and 8.6 per cent of ash. The ash contained from 58 to 62.35 per cent of phosphoric acid. The nitrogen and phosphorus were mainly in the form of nucleo-proteins and lecithin. The percentages of both nitrogen and phosphorus in the cell increase with age (81).

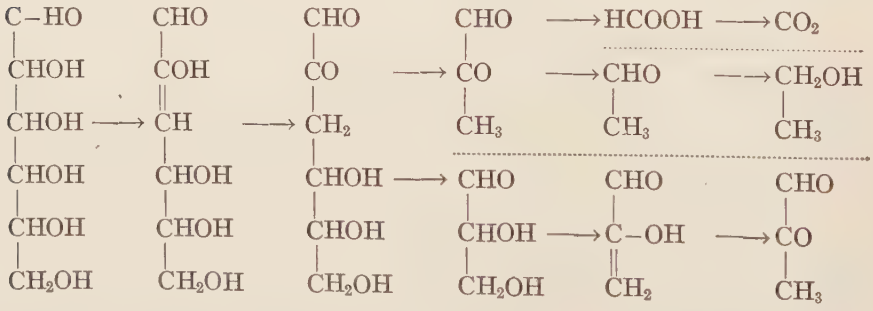
The most complete analyses of the *Azotobacter* cells, so far reported, show (147) them to contain, when grown on dextrin agar and rapidly dried at 30°C., 6.63 per cent of water, 4.12 per cent of ash, and 12.92 per cent of protein. The protein is similar to other plant proteins. It contains 10 per cent of

ammonia nitrogen, 26.5 per cent of diamino-nitrogen, and 60 per cent of monoamino-nitrogen. The quantity of lysine present is very high, but the histidine is present only in traces.

Krzemienwski (113) states that *Azotobacter* produces no hydrogen or other combustible gases in its metabolism, but according to Stoklasa (187) it does and in the presence of nitrates it produces ammonia and nitrites. Moler (142) claims that during its life, *A. chroococcum* separates no soluble compounds and it is only after death that it furnishes nitrogen to higher organisms. Nor are their bodies readily broken down by proteolytic enzymes. Both *A. agilis* and *A. vinelandii* separate a soluble nitrogen compound. The protein compounds so formed in soil are quickly broken down by other bacteria (42). Remy (165) considers the nitrogen fixed by *Azotobacter* in a readily available form for plant assimilation. Beijerinck found that 50 per cent of the total nitrogen in *Azotobacter* cells when supplied to the soil is nitrified in about seven weeks. None of the *Azotobacter* so far studied produce nitrates in the media (90).

Turning now to the breaking down of the carbohydrate, we find that the organisms produce ethyl alcohol (187), glycocoll, acetic acid, butyric acid, lactic acid, carbon dioxide and hydrogen. The quantity and quality of the different products vary with the species and with the carbohydrate used (112, 132).

It is likely that many of the end-products have not yet been determined, for Stoklasa (187) starting with 15.9 gm. of dextrose recovered 7.9 as carbon dioxide, 0.3 as ethyl alcohol, 0.2 as formic acid, 0.7 as acetic acid, 0.2 as lactic acid, but could not trace the remaining 6.6 gm. The organisms are extremely active when growing under appropriate conditions, for 1 gm. weight of *Azotobacter* has evolved no less than 1.3 gm. of carbon dioxide in 24 hours (185). A great distinction between the *Azotobacter* and the other species is that the former decompose their sugar with carbon dioxide as the chief product, whereas the other species form large quantities of butyric acid. Some of these products may be accounted for as follows:



It is known that when sugars, such as glucose, levulose and mannose are acted upon by alkalies, there are produced a great many products, some of

which are formic, carbonic, oxalic, lactic, pyruvic, tartronic, malic, molonic, tartaric, rebonic, saccharic, and gluconic acids in addition to many other either more or less complex compounds. We can readily conceive that the *Azotobacter* bring about a somewhat similar reaction, the stages, however, being more nicely governed, because of enzymes. Many of the products would be oxidized to carbon dioxide and water with the liberation of energy necessary for the endothermic nitrogen reaction; others readily react with the formed nitrogen compound. We are completely in the dark as to what this first nitrogen compound is, but we know that the *Azotobacter* possess the power of changing nitrates or nitrites under appropriate conditions into ammonia. Up to date it has been impossible to detect nitrate formation; it is not impossible that nitrates are formed and utilized by intra-cellular enzymes. By using nitrates, nitrites or ammonia, we can offer a rough explanation of protein anabolism.

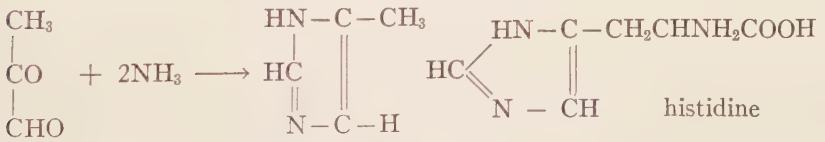
The endo-thermic reaction, $2N + 2H_2O = NH_4NO_2$, may take place and the ammonia thus formed may react with the decomposition products of the sugars—pyruvic acid, for instance—with the formation of alanine which Lipman considered as one of the first products:

$$(CH_3 - CO - COOH + NH_3 = CH - CHN - COOH + H_2O)$$
$$(CH_3 - CNH - COOH + H_2 = CH_3 - CHNN_2 - COOH)$$

or with glyoxylic acid forming glycocoll:

$$(HCO - COOH + NH_3 = HCNH - COOH + H_2O)$$
$$(CHNH - COOH + H_2 = CH_2NH_2 - COOH).$$

By similar reactions other amino acids may be formed. Moreover, Windas and Knoop (208) have shown that methylimadazol may be produced from glucose and ammonia, presumably through the formation of pyruvic aldehyde and formaldehyde, which is nearly related to the amino acid, histidine:



The various amino acids may, through the intervention of proteinases, condense with the formation of dipeptides, thus:

$$CH_3CNH_2COOH + CH_3CHNH_2COOH =$$
$$CH_3CHNH_2CONHCHCH_3COOH + H_2O$$

By the continuation of this process and by condensing with phosphorus—and sulfur-bearing compounds, probably through the intervention of other enzymes, there may result the complex protein of the *Azotobacter* cell.

PIGMENT PRODUCTION BY AZOTOBACTER

Most species of *Azotobacter* produce pigments. These vary in color from brown to black of the *A. chroococcum*, to a yellow or orange of the *A. vine-*

landii. The pigmented film usually develops on the culture media in from 3 to 7 days (105). It is formed by *A. chroococcum* earlier and in more abundance where old brownish cultures are used as the inoculating material. It is produced and retained within the bacterial cell; it occurs in neither the capsule nor the medium (86). The pigment produced by *A. chroococcum* is most pronounced when a dextrin agar medium to which calcium carbonate is added is kept at a temperature of 30°C. under well-aerated conditions. According to Jones (86), it is produced only when there is a lack of suitable available nutrient material and when organisms in the pigment area have ceased to multiply. The color of the pigment is intensified if nitrates (171) are added to the medium in which the organism is growing. The non-pigmented strains apparently fix nitrogen just as readily as do those which have not lost the power of forming pigments.

The pigment from *Azotobacter chroococcum* is insoluble in water, alcohol, ether, chloroform, benzol, and carbon bisulfide (149). It dissolves in alkalies, undergoing decomposition with the formation of a dark brown solution. Sackett (171) maintains that the peculiar brownish color which is characteristic of certain "nitre spots" of some soils is due to the pigment produced by *Azotobacter*. Such soils are high in nitrates and alkalies which would dissolve the pigments from the body of the organism. But Omelianski and Sswewrowa (149) are of the opinion that, although in some cases the dark color of vegetable soil may be due in a measure to the action of these microorganisms, it would be a mistake to attribute it to this factor alone. Furthermore, it has recently been proved (182) that the brown color of the "nitre spots" is due to solvent and decomposing action of the nitrates on the colored organic compounds of the soil, for they may be produced at will in a rich greenhouse soil with an excess of sodium nitrate, and this too in soils which have been rendered sterile with a saturated solution of mercuric chloride.

MORPHOLOGY OF THE NITROGEN-FIXING ORGANISMS

Of the many different bacteria which have been isolated and proved to have the ability to assimilate free nitrogen, *Clostridium pasteurianum* may be taken as a type of the anaerobic and *Azotobacter chroococcum* as a type of the aerobic.

Clostridium pasteurianum (211) is a short thick rod, from 1.2 to 1.3 μ in diameter and 1.5 to 2 μ long, in the young cells; the older spore-bearing cells take on a spindle shape. The bacteria stain a violet brown with iodine. The spores when ripe are 1.6 μ long and 1.3 μ broad and often lie in a roughly triangular covering. The ripe spore escapes through the wall of the mother in a longitudinal direction. Their germination is polar.

Azotobacter chroococcum occurs ordinarily as diplococci or short rounded rods 1 to 2 μ thick and 1.5 to 3 μ long, and according to Prazmowski (154) the microorganism first presents itself in its vegetative stage as a bacterium in the

fruiting stage as a micrococcus, and possesses a nucleus which functions in the same way as that of higher animals. In the resting stage the nucleus assumes a globular form, having a strongly refractive nucleolus with clearly differentiated boundary layers. The ndividuality of the nucleus appear to be practically lost at times, because of its relation to the cytoplasm. The division of the nucleus marks the first stage of cell division. According to Bonazzi (15) the organism shows peculiar granulations apparently not related to reproduction. These take the basic dyes and are neither fats, glycogen, starch nor chromatin, but appear to be of a metachromatic nature and seem to have their genesis in the nucleus. Their disposition in the cells is not constant but changes in different individuals. Involution forms occur and cell division is preceded by a simple form of mitosis (138). Some, but not all, varieties have been observed to form spores (131). The volutin bodies within the organism increase in number and size when the organisms are grown on media rich in nitrates. Hills (77) suggests that they may have some relation to nitrogen-fixation, but his results appear to oppose this view; whereas the addition of nitrates to a media greatly increased the reproduction, it very materially decreased the physiological efficiency of the organism. It seems, therefore, more likely that they are reserve protein material.

Löhnis and Smith (133) have recently observed that *Azotobacter*, in common with many other bacteria, pass through a life cycle which is not less complicated than those of other microorganisms. Under certain conditions they pass over into an amorphous or "symplastic" stage, appearing under the microscope either as an unstainable or a readily stainable mass without any easily distinguishable organization, which, if not discarded as dead, later gives rise to new regenerative forms. They multiple not only by fission, but by the formation of gonidia.

METHODS

Clostridium pasteurianum grows readily in a vacuum on carrots. The organism also grows on sliced potatoes, but ordinarily is grown in an aqueous solution containing 1 gm. K_3PO_4, 0.5 gm. $MgSO_4$, 0.01 to 0.02 gm. NaCl, $FeSO_4$, and $MnSO_4$, and 1.0 gm. $CaCO_3$, and 10 to 15 gm. of a suitable carbohydrate in 1 litre of water. One method used by Winogradsky in isolating *B. Clostridium pasteurianum* was to add garden soil to a non-nitrogenous solution and to allow a stream of nitrogen gas to pass through the solutions, after which several successive transfers were made into similar media. The final culture, after *B. Clostridium pasteurianum* had formed spores, was heated to 80°C.

The organism ferments certain carbohydrates with the formation of butyric acid acetic acid, carbon dioxide, and water. When grown in nutritive solution devoid of combined nitrogen, it assimilates atmospheric nitrogen, although in pure cultures it is an anaerobe. In nature it occurs in connection with two other bacteria which do not possess the power of fixing nitrogen, but

the nitrogen requirements of which are small. When in conjunction with these organisms *Clostridium pasteurianum* has the ability of growing in the upper layers of soil and of assimilating free nitrogen.

Azotobacter chroococcum grows readily on solid or liquid media, one of the best being:

	per cent
Monopotassium phosphate neutralized to phenolphthalein by sodium hydroxide	0.02
Magnesium sulfate	0.02
Sodium chloride	0.02
Calcium sulfate	0.01
Ferric chloride (1 per cent solution) 2 drops per 100 cc.	
Mannite	1.00

The organism is readily isolated by seeding this medium with soil. When the characteristic membrane forms, it is transferred by dilution to a similar medium containing agar in which the characteristic brownish black colonies form readily.

On mannite agar the colonies first appear as milky white glistening drops, round and convex, which under a low magnification show a coarsely granular structure extending to the margin. The colonies rapidly increase in size, and after a week or more become brown at the center with concentric rings alternating dark and white to the circumference and darker streaks radiating from the center outward. In old cultures, where the agar has partly dried up, the cells are often united in sarcina-like packets; the cell walls are much swollen and the contents are aggregated to a small ball at the center. At the same time giant cells, both round and elongated and filled with oil drops, can be seen. Often too a number of involution forms are seen, drawn out with long threads and false septa (2). By successive dilutions and transfers, it may be obtained in pure culture, although at times considerable difficulty is experienced in freeing it from a small organism—*B. radiobacter*.

Several different methods have been used for studying its nitrogen-fixing powers:

(a) Seeding into 100 cc. of the medium given above and after a certain time determining the nitrogen.

(b) The use of the same medium, but the addition of sufficient sand for the formation of sand slopes on which the organism can grow.

(c) The addition of a definite quantity of a carbohydrate to a soil and the incubation of this.

Each of these methods has its value. The first is much more readily handled in the final Kjeldahl determination, but the others give much higher results.

Freudenreich (47) found that when *Azotobacter* are grown upon gypsum, the gain in nitrogen is considerably in excess of that assimilated in the liquid media. Krainski (106) found *Azotobacter* to utilize from 100 to 200 gm. of sugar in the assimilation of 1 gm. of nitrogen when grown in solution, but

when grown on sand it required only 11 to 30 gm. for the same fixation. Many other workers have noted similar variation when grown in the soil. Where the organisms have been grown on gypsum or soil, we may attribute the stimulation to certain soluble constituents, yet this explanation scarcely seems plausible when considered in relation to sand cultures. Three strains of *Azotobacter* were grown in Ashby's mannite solution and sand (nearly pure silicon dioxide) to which Ashby's solution was added, with the following results:

	NITROGEN FIXED IN ASHBY'S SOLUTION	NITROGEN FIXED IN SAND
	mgm.	*mgm.*
Azotobacter A	6.86	22.61
Azotobacter B	5.00	12.60
Azotobacter C	6.44	16.80

Moreover, arsenic is very toxic in the solution, whereas when added to the soil or to pure quartz, in small quantities, it stimulates. Although the total quantities of nitrogen fixed under the two methods differ greatly, the relative efficiency of the organisms is about the same in both cases. In the testing of soils the same results are obtained, as may be seen from the following results, which is the average for several hundred determinations made on different soils by the two methods.

DEPTH OF SAMPLE	NITROGEN FIXED IN	
	100 gm. of soil + 1.5 gm. of mannite	100 cc. of Ashby's solution containing 1.5 gm. of mannite
	mgm.	*mgm.*
First foot	5 28	2.11
Second foot	2.42	0.77
Third foot	1.55	0.58

Although the greater aeration in the sand and soil culture probably plays a great part, there is little doubt that the colloids also assist.

RELATION OF AZOTOBACTER TO OTHER ORGANISMS

In the early study of nitrogen-fixation, the view was held that algæ growing on or near the surface of soil are able to fix nitrogen. Frank (44) in 1888 had observed such a growth on sand exposed to light and found that the soil showed a considerable increase in nitrogen. In 1892 Schloesing and Laurent (174) proved, both by determining the nitrogen fixed by a soil in a closed vessel and by observing the diminution of the nitrogen gas in the enclosed air, that a soil exposed to light gains in nitrogen if algæ are allowed to grow on the surface, and that the gain is confined to the upper few millimeters. They

did not, however, employ a pure soil or pure cultures of algæ. Kossowitsch (104), working with pure cultures of two green algæ, found no fixation, but observed a considerable increase of soil nitrogen when they were grown with soil bacteria. Later, Krüger and Schneidewind (110), employing pure cultures of many other chlorophyceæ, obtained no nitrogen-fixation. Hellriegel and Deherain had found a large increase in the nitrogen content of sand in pots when exposed to the light, which was always accompanied by a development of algæ. In the light of such results, the conclusion has been reached that algæ alone cannot assimilate free nitrogen, but only in concurrence with soil bacteria, the former producing carbohydrates which are used by the latter as a source of energy for the nitrogen-fixation. Heinze (73) actually observed rapid fixation of nitrogen when cultures of algæ were inoculated with *Azotobacter* or other nitrogen-fixing organisms. Stoklasa (138) found that *Azotobacter* are especially abundant in soils having a vigorous growth of blue-green algae. *Azotobacter* are often absent from virgin soils, but are always found in such soils when there is a vigorous growth of algæ (191). Bottomley (18) claims that both *Azotobacter* and *Pseudomonas* live in true symbiosis with cycas. It, therefore, appears certain that the nitrogen-fixing powers of *Azotobacter* are greatly enhanced when growing with algæ, but the exact rôle played by each is yet to be explained. This offers a rich and inviting field for research.

Nor is it alone in combination with algæ that these organisms may grow and thus be benefited. Beijerinck and Van Delden (9) early recognized that an apparent symbiosis exists between *Azotobacter* and other bacteria, and that the nitrogen fixed is considerably greater in the mixed than in the pure cultures. This symbiosis, though in many cases beneficial to *Azotobacter*, is not essential for nitrogen-fixation (123). Radiobacter, with which the *Azotobacter* are usually associated, have only slight nitrogen-fixing powers (187), yet they increase the nitrogen-fixing powers of *Azotobacter* (112). The carbohydrates disappear more rapidly from mixed than from pure cultures and with a greater fixation per gram of carbohydrate utilized (17). There is also a greater fixation when two strains of *Azotobacter* are grown in conjunction with each other. This is especially marked in an aqueous solution of mannite (65). Results have been reported (16) where *Azotobacter* fixed twice as much nitrogen in the presence of *Pseudomonas* as when grown alone.

The manner in which this mutual benefit is exerted is not clear. In some cases it may be due to the associated organism rendering more available the carbonaceous material.

Omelianski and Salunskov (148) offer the following explanation concerning the association of aerobic and anaerobic nitrogen-fixers:

> The synergetic activity of nitrogen-fixing and accompanying microbes, is both in laboratory experiments and under natural conditions (cultivable stratum of the soil) of a different character according to the properties of the species taking part in the process and their environment; in both cases the function of the satellite organism seems to consist in

fixing the oxygen of the air and creating the anaerobic environment for *Clostridium pasteurianum*. The species added to the cultures of nitrogen-fixing microbes sometimes supply the compounds of carbon needed for the process of fixing nitrogen as energetic substance. In the case of the combination: *Azotobacter* + *Clostridium pasteurianum*, the function of the former is not confined to fixing the oxygen of the air only, and consequently to creating an anaerobic environment for the *Clostridium*. But this combination is also useful inasmuch as it destroys the injurious products of disassimilation created by the second (chiefly butyric acid) and maintains the action of the environment. (*Azotobacter* is alkaligenic and the *Clostridium* acidogenic.)

The satellite species may also unfavorably affect the nitrogen-fixing microbe, either through products of assimilation or by consumption of the carbon compounds needed by this microbe for nitrogen-fixing. The energetic fixation of oxygen by the satellite aerobic species creates conditions favorable to the development of *Clostridium pasteurianum*, but at the same time hinders the growth of the *Azotobacter*, which is necessarily aerobic.

The form endowed with the maximum vitality and at the same time the most common form in which combination of the nitrogen-fixing organisms takes place in the upper soil strata is that of symbiosis between the aerobic and anaerobic nitrogen fixers, principally between *Azotobacter* and *Clostridium pasteurianum*. In spite of the opposite properties of the two species, their synergetic activity in the upper strata of the soil results in a harmonious mutual development producing the maximum economy in consumption of energetic substances.

So far, little has been done to determine the relationship of *Azotobacter* to the higher plants, but it is interesting to note that Beijerinck (8) has observed a distinct relationship between the distribution of the organism and leguminous plants. Fischer (42) suggests that some nitrogen-fixing bacteria presumably exist first as saprophytes, then as exoparasites in loose combination with green plants, then as endoparasites. Finally they develop the true symbiosis of root nodule bacilli. Hopkins (82) has questioned whether there may not be a relationship between the legume bacteria and *Azotobacter*.

THE INFLUENCE OF WATER

Azotobacter are very resistant to drying; they may be dried for a considerable time in a desiccator over sulfuric acid. Pure cultures are just as resistant to drying as are mixed cultures (89). This would vary some with the media in which the bacteria are dried, for the survival of non-spore-bearing bacteria in air-dry soil is due, in part, to the retention by the soil of moisture in the hydroscopic form. This, however, is not the only factor, for the longevity of bacteria in a solid is not directly proportional to its grain size and hygroscopic moisture. Giltner and Langworth (53) found that bacteria resisted desiccation longer in a rich clay loam than in sand. Furthermore, if bacteria are suspended in the extract from a rich clay loam before being subjected to desiccation in sand, they live longer than if subjected to desiccation after suspension in a physiological salt solution. Because of this, they consider that soils contain substances which have a protective influence upon bacteria subject to desiccation.

Lipman and Burgess (117) have found that many soils manifest a vigorous nitrogen-fixing power even after being air-dried and kept in stoppered museum

bottles for periods varying from 5 to 20 years. In some cases the fixation was equally as high as in freshly-collected samples. The organisms from such soils are more easily attenuated than are other organisms which have not been so dried (207). The tendency is for soils gradually to decline in nitrogen-fixing power on drying. This may manifest itself as early as the second week.

During the periods of drying, the organisms are inactive, as they require moisture for growth and reproduction. For maximum nitrogen-fixation a definite moisture content is needed. Warmbold (203) found the optimum moisture content to be 20 per cent. When it was below 10 per cent there was no nitrogen fixed, and in some cases there was a decided loss of nitrogen. Krainski (105) allowed soil with varying moisture content to stand for some time and then inoculated it into mannite solutions and obtained maximum fixation in the soils containing fairly small quantities of water. Later, however, he decided that soil should be damp—but not wet—and well aerated for maximum nitrogen-fixation. The water requirements vary with different soils. As a general rule, the higher the humus content of the soil, the more water will be required for optimum nitrogen-fixation (108). The quantity of water present may, however, become so great that it may kill all *Azotobacter* in addition to stopping nitrogen-fixation (42).

An insufficient supply of moisture checks both nitrification and nitrogen-fixation (34). This occurs in some soils when the water content has been reduced to 16.5 per cent. This again varies with the soil, for Schloesing (173) found bacterial activity less in fine-grained soils than in lighter, coarse-grained soils. A difference in moisture content of 1 per cent, according to Defert and Bollinger (32), is sufficient to produce a marked change in the oxidation going on in the soil.

The moisture requirement of the nitrogen-fixing bacteria, according to Lipman and Sharp (119), is more nearly that of the ammonifying than of nitrifying organisms. In a sandy loam it was found to vary between 20 and 24 per cent of moisture in the soil. At the higher percentages of moisture up to 24 per cent the anaerobic nitrogen-fixers are most active, but the action of the aerobes is slightly depressed. Thus, in many soils two maxima of nitrogen-fixation occur, depending upon whether the conditions are favorable for the anaerobic or aerobic organisms.

Traaen's results (195) differ from Lipman's in showing only the one maximum, as is seen from the following, which gives the milligrams of nitrogen fixed in 100 gm. of soil.

TEMPERATURE	5 PER CENT H_2O	10 PER CENT H_2O	17.5 PER CENT H_2O	25 PER CENT H_2O	30 PER CENT H_2O
13°C.	0.1	1.5	11.2	13.4	5.4
25°C.	1.9	1.9	13.2	16.6	15.5

He used a loam soil with a maximum water capacity of 27.4 per cent. It is quite evident from his statement that anaerobic organisms played a prominent part in the fixation at the higher moisture contents.

Since the carbohydrates disappeared much more rapidly in the soils containing the greater quantities of water, it is quite possible that greater quantities of nitrogen per gram of carbohydrate consumed are fixed where the smaller quantities of water are applied. This, together with the different methods used by the several investigators, would explain the apparent discrepancy in their results.

In a series of pot experiments in which a calcareous loam receiving various amounts of water was used, the author (58) found the moisture content for maximum nitrogen-fixation to lie between 15 and 22 per cent. These results also bring out the two maxima which were first noted by Lipman. These soils were kept at the various moisture contents for four months. All were then incubated at 28°C. for 21 days with a moisture content of 20 per cent.

TREATMENT	NITROGEN FIXED
per cent	*per cent*
12.5	100
15.0	108
17.5	102
20.5	104
22.5	108

In this soil the optimum for the aerobes would appear to be at 17.5 per cent and that for the anaerobes 22.5 per cent or higher.

When too large a quantity of water is applied there is a tendency to depress the total nitrogen fixed, as is illustrated by the following results in which various quantities of water were applied to a soil throughout the year under field conditions (59).

37.5 inches of water applied during summer; 1.4 mgm. of nitrogen fixed in 100 gm. of soil.
25.0 inches of water applied during summer; 2.1 mgm. of nitrogen fixed in 100 gm. of soil.
15.0 inches of water applied during summer; 8.5 mgm. of nitrogen fixed in 100 gm. of soil.
No water applied during summer; 3.5 mgm. of nitrogen fixed in 100 gm. of soil.

The maximum for anaerobic conditions does not appear in these results probably because the soil did not become filled with water and because under field conditions the water rapidly drains away or is evaporated.

TEMPERATURE

Berthelot (13) early recognized that the biological gain of nitrogen in soils is dependent upon a suitable temperature. He found nitrogen-fixation to occur best at summer temperatures between 50° and 104°F. The process was

immediately stopped on heating to 230° F. Later Thiele (193) maintained that although *Azotobacter* possess the ability to fix small quantities of nitrogen under laboratory conditions, the temperature would be unfavorable under field conditions. Heinze (72), however, found that although the nitrogen-assimilating organisms are most active at a temperature between 20°C. and 30°C., they nevertheless fix appreciable quantities at temperatures as low as 8 to 10°C. Still more recent work (111, 134) has shown the optimum temperature to be 28°C., and the limits of activity of *Azotobacter chroococcum* to lie between 9°C. and 33°C. The actual quantitative variation in nitrogen fixed is seen from results reported by Löhnis (128). He inoculated 100 cc. of a 1-per cent mannite soil extract with 10 gm. of soil and obtained the following fixation at the various temperatures:

10° TO 12°C.	20° TO 22°C.	30° TO 32°C.
3.15 mgm. nitrogen	4.55 mgm. nitrogen	4.27 mgm. nitrogen

Better fixation at a lower temperature is noted when the soil is incubated and the gain in nitrogen determined directly. Koch (95) obtained fixations of 3 mgm., 11 mgm., and 15.5 mgm. of nitrogen in 100 gm. of soil when incubated with a carbohydrate at 7°C., 15°C., and 24°C., respectively. Traaen (195), using a loam soil with a maximum water-holding capacity of 27.4 per cent, obtained nearly as great a fixation at 13°C. as at 25°C. when the optimum moisture content was maintained. This is seen from the following:

TEMPERATURE	NITROGEN FIXED IN 100 GM. OF SOIL				
	5 per cent H_2O	10 per cent H_2O	17.5 per cent H_2O	25 per cent H_2O	30 per cent H_2O
	mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*
13°C.	0.1	1.5	11.2	13.4	5.4
25°C.	1.9	1.9	13.2	16.6	15.5

A temperature, favorable even though not ideal for nitrogen-fixation, would occur in soils under natural conditions. The temperature of soil in Utah during the months of September averaged 14°C., with a minimum of 10°C. and a maximum of 17°C. During June, July and August the mean temperatures would be much greater.

The mean daily temperatures of the soil for Bismarck, North Dakota; Key West, Florida; and New Brunswick, New Jersey; for the months of June, July, August and September were 18°C., 28°C., and 24.5°C., respectively. From this it is evident that during a considerable period of each year an arable soil has a temperature high enough for moderately rapid nitrogen-fixation.

Although it is generally maintained that there is no nitrogen-fixation in soils during the winter months, cold or even freezing does not injure the or-

ganism; for the cooling of a soil, even to the freezing point, increases its nitrogen-fixing powers (24). This is probably due to the suppression of competing species and to the establishment of a new flora. The same is true when the soil is heated, as may be seen from the results given below (56).

TEMPERATURE	NITROGEN FIXED
deg. C.	*mgm.*
Normal	5.11
50	9.00
55	14.14
60	16.38
65	14.42
70	13.02
75	11.34
80	12.66
85	10.36

This soil had been autoclaved and then inoculated with a soil extract which had been heated to the temperature indicated. The stimulation could not, therefore, have been due to the heat rendering more of the plant-food in the soil available. The results indicate that many of the organisms which take part in nitrogen-fixation are highly resistant to heat. It is significant that the greatest stimulation is exerted in a soil which had been inoculated with solutions heated just above the temperature which Cunningham and Löhnis (31) found to be the thermal death-point of soil protozoa.

LIGHT AND OTHER RAYS

As a class, bacteria are sensitive to light, but the extent to which they can withstand it varies, among other things, with the conditions of exposure and the specific organism. Unfortunately, we have but fragmentary information concerning the effect of light upon azofiers, but what we do know would lead us to believe they are more resistant than many microorganisms—probably more so than the many other soil bacteria. Berthelot (13) recognized that nitrogen-fixation in the soil occurred both in daylight and in darkness, though more freely in the light. Jones (86) found many *Azotobacter* to be alive in a small Petri dish of dried soil that had stood in the laboratory in front of a south window for two years. They can withstand the direct action of the violet and of the longer ultra-violet rays for five minutes (190), but are killed in much less time by the shorter ultra-violet rays. They are more resistant even to these than are many other species.

The fixation of elementary nitrogen by *A. chroococcum* is distinctly increased when the air is activated by pitchblende. Somewhat better results are obtained with weak than with stronger radio-active intensity.

AERATION

Under field conditions there is a mixed flora consisting of the anaerobic and aerobic nitrogen-fixing microorganisms. A soil condition which would be ideal for one species might be unfavorable for the other. It has already been pointed out that there are two maxima of nitrogen-fixation in soils, depending upon the moisture content. This is illustrated in figure 1.

Although it is usually conceded that nitrogen-fixation is most rapid when soils are well aerated, this may not always be the case. Concerning this Murray (145) reports the following results:

KIND OF SOIL	NITROGEN FIXED	
	Aerobic conditions	Anaerobic condition
	mgm.	*mgm.*
Greenhouse soil	0.84	8.50
Loam soil	3.08	5.29
Clay soil	0.84	4.69

This condition must be attributed to a great difference in the physiological efficiency of the two groups found in these particular soils and not to a lack of aerobic nitrogen-fixing organisms, for more than ten times the number of organisms developed on nitrogen-poor media from these soils under aerobic as under anaerobic conditions.

SEASON

Berthelot (13) was unable to show any gains in nitrogen of his soils during the winter, but Koch (94) found a considerable increase during the season in soils which were kept in a heap and shoveled over from time to time. Löhnis (127) observed that *Azotobacter* membranes are more readily obtained in winter than in summer. He later found that the nitrogen-fixing power of soil varies from month to month throughout the year, there being two maxima —one in spring and another in autumn (128). The extent of the variation noted may be seen from the following:

1903–1904	*March*	*May*	*July*	*September*
	100*	121	50	100
1907	*April*	*May–June*	*July–August*	*October–November*
	100*	133	69	122

* The relative numbers are based on the spring months as 100.

Green (60) found nitrogen-fixation in 1 per cent mannite solution to be low during August, September and April. In other months he noted a fairly constant fixation of about 10 mgm. of nitrogen per gram of mannite. He also noted a marked yearly variation in the nitrogen fixed during July and August.

Walton (201) found nitrogen-fixation lowest in Indian soil between October and January and highest between June and September. This corresponds with moisture and temperature changes. Peterson (153) has found that although the nitrogen-fixation of Utah soils is highest from June to September, the number of types of *Azotobacter* occurring in the soil was greatest in May. Moll (143) goes so far as to maintain from his work that the season of the year is the principal factor in determining the biochemical transformations in soils. This would appear to be especially true as regards nitrogen-fixation.

CROP

Heinze (72, 74) called attention to the fact that the fallowing of the soil increased its nitrogen-fixing power. This could be due to better aeration, moisture, temperature, etc., and not to any depressing influence exerted directly by the plant. Most experiments which consider plant and bacterial activity could be interpreted in this light. Hiltner (78) maintains that the free nitrogen-fixing bacteria are stimulated in their activities by the growing plant roots. There may be considerable truth in this, for here the higher plants are rapidly removing from the solution the soluble nitrogen compounds. In this case, the nitrogen-fixing organisms would be forced either to compete with the higher plant for the soil nitrogen or else to make use of their ability to live upon the atmospheric nitrogen. It is certain that different cultural methods vary sufficiently with crops to influence profoundly a soil's nitrogen-assimilating properties, for the *Azotobacter* occur more widely distributed in cultivated than in virgin soil (74). The analyses of hundreds of samples of cultivated and virgin soils in Utah (55) have in nearly every case shown the virgin soil to have a low nitrogen-fixing power as compared with the cultivated soil. This was the case even where the soil was incubated without carbohydrates and the nitrogen determined directly. The average results for many determinations were as follows:

	mgm. of nitrogen fixed
Virgin soil	6.99
Cultivated	14.28
Wheat	11.83
Alfalfa	12.24
Fallow	22.81

Because the fallow soil had received considerable manure, the results are undoubtedly high. It would, however, be possible to fallow or crop soils so continuously that extremely small quantities of plant residues would be returned to the soil, under which conditions there might be a decrease in nitrogen-fixation. The conditions of moisture and aeration are much more nearly ideal in a fallow soil than in a cropped soil. It is just possible that the high fixation noted where wheat is grown continuously may be due to the method

in vogue in the arid districts of leaving the greater part of the straw on the soil. This would act as a readily assimilable carbonaceous material for the *Azotobacter*. Welbel and Winkler (205) have found that fallowing not only increases the assimilable nitrogen but also the available phosphorus of the soil, a liberal supply of which causes the *Azotobacter* to utilize its energy more economically. That the increased nitrogen-fixation noted when soils are cultivated is not confined to the arid soils, is seen from the recent work of Reed and Williams (163). Brown's work (22) indicates that crop rotation increases the nitrogen-fixing powers of a soil.

CLIMATE

It has been maintained for a long time that there is a close correlation between the chemical, physical, and biological transformations going on in a soil and the climatic conditions, but there was nothing definite on this subject until the highly interesting work of Lipman and Waynick (120) appeared. They found a definite relationship between climate and the nitrogen-fixing powers of a soil. Removal of California soil to Kansas increased the vigor of the *Azotobacter* flora and especially that of *A. chroococcum*. It increased the nitrogen-fixation by 50 per cent over that attained by the same soil in California. Similar results were obtained in California soils removed to Maryland. Kansas soil taken to California lost its power to produce a membrane in mannite solution, the *Azotobacter* flora became rather feeble, and the nitrogen-fixing powers of the soil were greatly reduced. The removal of the Kansas soil to Maryland increased the vigor of the *Azotobacter* and induced a higher fixation of nitrogen. The Maryland soil in California diminishes in nitrogen-fixing powers, but not in so great a degree as does the Kansas soil. This also happened when the Maryland soil was taken to Kansas. The bacterial flora of a soil is, therefore, dependent upon climatic conditions which affect many of the other properties of a soil.

RELATIONSHIP OF AZOTOBACTER TO NITRATE ACCUMULATIONS

The fact that certain spots in western cultivated soil were very rich in nitrates wa first observed by Hilgard (76). This, he attributed to the rapid nitrification of the organic matter of the soil in the warm arid climate of the West when the moisture limit was removed by irrigation.

A number of years later Headden (69) noted these "nitre spots" in a number of Colorado soils, but he attributed it to the fixation of atmospheric nitrogen by the non-symbiotic bacteria which find in the western soils ideal conditions for growth and for rapid nitrogen-fixation. This conception has been further amplified by Headden (70, 71) and also Sackett (171). In this work it is assumed that the *Azotobacter* not only fix the nitrogen but also produce the nitrates. It has been proved, however, that these organisms do not produce nitrates (90).

Moreover, there are a number of other vital objections to this theory. (a) Lipman (125) has shown that for the fixation of the quantity of nitrogen which Headden maintains to have occurred, it would require from 1000 to 2000 tons of carbohydrates. There is no such visible supply of energy in these soils. True, many of these soils have a rich algae flora, but it has not been proved that this will furnish a sufficient supply of available energy. (b) The average amount of nitrogen fixed in 32 samples collected in the nitrate region was 7.4 mgm. (171), and the average nitrogen fixed in 31 samples of dry-farm alkali-free soil in Utah was 12.2 mgm. (55); yet there is no accumulation of nitrates in these latter soils. (c) The quantity of soluble salts occurring is often sufficient to stop the activity of all nitrogen-fixing organisms, if not to kill them (118). (d) The quantity of nitric nitrogen and of chlorine in any given "nitre spot" varies in the same spot from year to year or from period to period within a year (181). (e) The country rock adjacent to the nitrate accumulations, and which has contributed to the soil formation contains abundance of nitrates to account for the accumulations noted (182). (f) Soils having a similar physical appearance may be produced in the laboratory in the absence of bacteria. Because of this, we must conclude that the accumulation of nitrates in spots in western soils have their origin as do other accumulations of soluble salts found in the soil and not in the fixation in place by bacterial activity.

THE ACTION OF AZOFIERS ON PLANT-FOOD

It is quite evident that *Azotobacter* in their metabolism transform soluble inorganic soil constituents into either soluble or insoluble organic forms. This is especially true of phosphorus which is found in the ash of these organisms in such large quantities. The phosphorus, on the death of the organism, would be returned to the soil in a readily-available form, for Stoklasa has found that 50 per cent of the nitrogen of these organisms is nitrified within six weeks, and there is no reason for believing that the phosphorus would be liberated much more slowly. Then there is the possibility that many of the constituents of the bacterial cell may become available through the action of autolytic enzymes without the intervention of other bacteria (126).

It is further evident that an organism, which possesses the power when growing under appropriate conditions of generating 1.3 times its own body weight in carbon dioxide during 24 hours (185), must greatly change the composition of the media in which it is growing. Water charged with carbon dioxide is a universal solvent and will attack even ordinary quartz rock. Granite and rocks related to it are rather quickly attacked with the liberation of potassium and other elements. Likewise, it would act upon the tricalcium phosphate of the soil with the formation of more readily soluble phosphates, for this substance is four times as soluble in water charged with carbon dioxide as it is in pure water.

$$Ca_3(PO_4)_2 + 2CO_2 + 2H_2O = Ca_2H_2(PO_4)_2 + Ca(HCO_3)_2.$$

Moreover, the nitrogen-fixing organisms form among other products formic, acetic, lactic, butyric and other acids. The kind and quantity of each depends upon the specific organisms and upon the substance on which they are acting. These substances are sure to come in contact with some insoluble plant-food which may be rendered soluble, for they have a high solvent power for the insoluble phosphates (180). The resulting salts of calcium would be further attacked by bacteria with the formation of calcium carbonate (54).

Whether these processes will give rise to an increase in the water-soluble plant-food of the soil will depend upon whether the products of the second, the analytic reactions, exceed the products of the first, the synthetic reactions. We must not lose sight of the fact that, although many of the organic phosphorus constituents may not be soluble in pure water, they may be more available to the living plant than are the constituents from which they were at first derived through bacterial activity.

This being the case, we may expect to find variations in the results reported from laboratory tests. Stoklasa (184) found that bacterial activity rendered the phosphorus of the soil more soluble, whereas Severin (176) in his early work found the opposite to be true. Others have found that the solvent action of bacteria for insoluble phosphates is in direct proportion to the acid secreted by the organism (172).

In a later work, Severin (177) obtained different results. He used three soils—one sterile, a second sterilized and inoculated with pure cultures of *Azotobacter*, and a third sterilized and inoculated with cultures of *B. radicicola* and *Azotobacter*. The solubility of the phosphorus increased 8 to 14 per cent over that in the sterile soil. The acid-producing organisms, because of the acid secreted and their intimate contact with the soil particles, possess the power of dissolving silicates (5). Moreover, since arsenic greatly stimulates nitrogen-fixation, there is a relationship between this increased bacterial activity and the form and quantity of phosphorus found in a soil (57).

The following results were obtained as an average of a great number of determinations. The addition of 16.0 mgm. of arsenic to a soil in the form of lead arsenate increased the nitrogen fixed in unit time 5.6 mgm. per 100 gm. of soil. It increased the water-soluble phosphorus in that soil 0.07 mgm., the 12 per cent hydrochloric acid-soluble phosphorus 5.8 mgm., and the organic phosphorus in the soil 1.3 mgm. per 100 gm. of soil. Now it is known that arsenic increases the activity of these organisms when applied to them even in the presence of an abundance of available phosphorus. It seems reasonable, therefore, to conclude that the excessive bacterial activity had slightly increased the water-soluble, the acid-soluble and the organic phosphorus of the soil.

Although the metabolic activity of *Azotobacter* gives rise to large quantities of phosphate solvents, yet these organisms transform phosphorus into organic phosphorus compounds less rapidly than do the ammonifiers (189).

SOIL INOCULATION

High hope was entertained that the nitrogen problem in agriculture had been solved, when Caron (25) announced that he had prepared a culture of bacteria which would enable non-leguminous plants to utilize free atmospheric nitrogen, provided certain precautions were observed. Many of the results which he reported on pot experiments were clearly in favor of the inoculated plant. Stoklasa (183) was one of the first to study in detail the commercial preparation "alinit" which was placed on the market as a result of Caron's work. His findings were fully as favorable as Caron's, but the work of others soon demonstrated that alinit neither in the laboratory nor in the field had the ability to fix nitrogen. When Beijernick discovered the free-living aerobic nitrogen-fixers, the hope that soil inoculation may be so perfected that it would be beneficial to crops was revived, and since that time many investigators have attempted to inoculate soil in order to increase its crop-producing powers, but usually with negative results. Stoklasa (188) has made great claims for soil inoculation. He found that soils, inoculated with *Azotobacter chroococcum* and adequately supplied with carbohydrates and lime, showed an increase in the number of nitrogen-fixing organisms, and also an increased yield both in quantity and quality of the crop. Stranak (191) also obtained a pronounced increase in the production of beets, grain, and potatoes on inoculating with *Azotobacter*.

There may be a decrease in the crop during the first year when carbohydrates and *Azotobacter* are added to the soil with a marked increase in crop during the second and third year. Even then, the soil may be left richer in nitrogen than it was at first.

Effect of dextrose and sucrose on the productiveness and nitrogen content of the soil (96)

CARBOHYDRATE ADDED PER 100 GM. OF SOIL	CROPS OBTAINED				TOTAL NITROGEN REMAINED IN CROP	TOTAL NITROGEN LEFT IN SOIL, SPRING OF 1906	NITROGEN AS NITRATES
	Oats, 1905		Sugar Beets, 1906				
	Dry matter	Yield of nitrogen	Dry matter	Yield of nitrogen			
					gm.	*per cent*	*parts per million*
None	100.0	100.0	100.0	100.0	0.5914	0.093	10
2 per cent dextrose	32.8	62.5	186.0	190.0	0.6814	0.105	17
2 per cent sucrose	33.3	58.7	179.0	195.0	0.6800	0.105	15
4 per cent sucrose	37.7	78.1	283.0	339.0	1.0092	0.119	37

It is often the case that the addition of starch to a soil during the first year retards plant growth. This injurious action (40) may be due to the augmented bacterial activity in the soil brought about by the carbohydrates which injure the roots of the plant by withdrawing oxygen and by forming hydrogen sulfide in the deoxygenated atmosphere of the soil through the reduction of sulfates by the bacteria.

The effect produced by the carbohydrate applications also varies with the season (64). If applied to the soil in the spring when the soil temperature is low and when other bacteria are more active than *Azotobacter*, the results are that they rapidly multiply and compete with the higher plants for the limited available plant-food. If, however, the carbohydrates are applied in the autumn directly after the removal of the crop, when the soil is warm, *Azotobacter* are active, with the result that sufficient nitrogen is fixed to produce an increased crop the following season.

If the same quantity of carbohydrates per unit of nitrogen fixed be required by the organism under natural conditions, as are found necessary in laboratory experiments, enormous quantities would be required for the fixation of any considerable quantity of nitrogen; but it is possible that in the soil they are more economical (100) with their energy or they may live in symbiosis (106) with other organisms which furnish them part of their carbon.

Many workers have noted either no effect (33, 123) or even a detrimental influence (124, 196) when soils are treated with the carbohydrates and then inoculated with *Azotobacter*. This may be due in a great measure to any or all of the following factors: (a) absence of a suitable environment, as temperature, moisture, aeration, food and alkalinity; (b) absence of a suitable host from which *Azotobacter* may obtain part of its carbon; (c) injurious effects due to the decomposition products of the carbohydrate added (96).

There is considerable interest in the work of Bottomley (17) who uses bacterized peat, or humogen. The bacterizing process consists of three stages: (a) treatment of peat with a culture solution of the special "humating" bacteria and an incubation of it at a constant temperature for a week or ten days, during which period soluble humates are formed; (b) destruction of the humating bacteria by sterilization with live steam; (c) treatment of this sterilized peat with mixed cultures of nitrogen-fixing organisms—*Azotobacter chroococcum* and *Bacillus radicicola*—and an incubation at 20°C. for a few days, after which it is ready for use.

Theoretically, there is much in this process which recommends it, for there is no abrupt change in environmental conditions for the organism added, as would be the case when added from laboratory culture. Moreover, they are added in enormous quantities and with a source of carbon which is not far different from that found in the soil. Russell (170), however, after carefully reviewing all of the experimental evidence on the subject, concludes: "There is no evidence that humogen possesses any special agricultural value. There is not the least indication that it is 50 times as effective as farmyard manure, to quote an often repeated statement, and there is nothing to show that it is any better than any other organic manure with the same nitrogen content." Furthermore, he concludes that there is no definite evidence that "bacterization" really adds to the value of peat.

The conclusion is evident that soil inoculation, in order to be successful, must be accompanied by the rendering of the physical and chemical properties

of the soil ideal for the growth of the specific organisms to be added. A few organisms placed in a new environment already containing millions can never hope to gain the ascendancy over the organisms naturally occurring in the soil, for they have been struggling for countless generations to adapt themselves to the environment and only those which are fitted have survived. The problem becomes even more complicated when we recall the findings of Lipman that the bacterial flora of a soil is in many cases entirely changed by climatic conditions. On this account, it would appear that ever to make soil inoculation a success the chemical, physical, and even the biological condition must be made suitable for the growth of the specific organism added. Furthermore, strains of the organism must be used which have been evolved under similar climatic conditions.

SOIL GAINS IN NITROGEN

It is well established that many forms of microscopic organisms possess the power of fixing nitrogen either when grown alone or in combination with other organisms of the soil. Many of these have been obtained in pure culture and their morphology and physiology carefully studied. The most favorable conditions for their maximum nitrogen-fixation in pure cultures in liquid solutions have been accurately determined. Some of the conditions requisite for their activity in soils are known, but on this phase of the subject there are many gaps in our knowledge and much work must yet be done before we can state definitely the part which they play in the economy of nature and before we can say which are the very best methods for increasing their usefulness. Nevertheless, it is interesting to consider the results obtained by a few workers.

Berthelot's early laboratory experiments (13) led him to believe that sands and clays may fix in a year from 75 to 100 pounds of nitrogen to the acre. In two exceptional instances he noted that nitrogen was fixed by sands at the rate of 525 pounds and 980 pounds an acre, but soils which contained fairly large quantities of nitrogen never made markedly rapid gains.

Thiele (193), on the other hand, maintained that while there is no doubt that *Azotobacter* possessed the power of fixing free nitrogen, under laboratory conditions, yet it is not certain that conditions would be such in soils for any gain of nitrogen due to the activity of these organisms. We have already seen, however, that the *Azotobacter* do not require as high a temperature for nitrogen-fixation in soil as he thought necessary. It is also certain that in most arable soil the temperature is sufficient during a large part of the year for a fairly rapid nitrogen-fixation by bacteria.

Krainskii (106) thinks that even better results should be obtained in soils than in pure solutions, for there the nitrogen-fixers grow in symbiosis with autotrophic organisms which form organic compounds available to the *Azotobacter*. In soils the nitrogen fixed is rapidly removed by other plants, because

of which the slowing-up process which becomes perceptible so early in laboratory experiments should not occur.

In addition to an optimum temperature and moisture content of the soil, the *Azotobacter* are dependent upon a supply of carbon for energy and inorganic nutrients for the building of cell protoplasm. Unfortunately, it is too often the case that under natural conditions those soils which are deficient in nitrogen are also lacking in available carbon, and especially in phosphorus, which are so essential for rapid nitrogen-fixation. Then there are the technical difficulties which the chemist encounters in determining the gain or loss of nitrogen which occurs in soils under natural conditions and which may be attributed to non-symbiotic nitrogen-fixation.

There are, however, several cases in which this has been measured with a fair degree of accuracy.

Lipman (198), in pot experiments carried on with a soil containing about 5000 pounds of nitrogen per acre-foot of soil, found a gain of more than one-third this amount in two short seasons. Much of this must be attributed to non-symbiotic nitrogen-fixation. To these soils had been applied solid and liquid manure, which furnished to the organisms readily-available supplies of energy and various necessary inorganic constituents. This fixation was not nearly so rapid where legumes were turned under as green manures.

Koch found a gain of from 0.093 per cent to 0.019 per cent in soil nitrogen during two seasons which must be attributed to non-symbiotic nitrogen-fixation. In addition to this there was a threefold gain in the nitrogen content of the crops—oats, buckwheat, and sugar beets—which must also be attributed to the action of *Azotobacter*.

Hall (63) noted an annual gain of 100 pounds of nitrogen on Broadbalk field at Rothamsted and 25 pounds on Grescroft field. He feels that much of this gain must be due to the action of non-symbiotic bacteria. Lipman (199) points out that the actual gains of nitrogen are even greater, for this does not take into consideration the various losses which are sure to occur even under the best of conditions. Hopkins (82) takes the stand that the apparent gain is due in a large measure to drifting dust and plant residues coupled with the difficulty of obtaining representative samples of soil at the two different periods. When all of these factors are considered the evidence points to a gain of nitrogen through bacterial activity.

The analysis of a great number of soils in Utah (56) showed that the average nitrogen content of the soil which had grown wheat and other non-leguminous plants for from 20 to 50 years was 0.2009 per cent, whereas adjoining virgin soil on the average showed only 0.0984 per cent of total nitrogen. The evidence is very strong that considerable nitrogen has been added to these soils by microscopic organisms, for:

(a) In nearly every case the cultivated soil fixed much more nitrogen in the laboratory than did the virgin soil. This was the case when the soil was incubated with or without the addition of carbonaceous material.

(b) There is a richer nitrogen-fixing bacterial flora in the cultivated than in the virgin soil.

(c) The conditions of moisture, alkalinity and food constituents in the soil were ideal for rapid nitrogen-fixation, and the temperature of the soil was high enough during a considerable part of the year for the growth of *Azotobacter*.

(d) The cultivation of the soil would increase aeration and available phosphorus in the soil.

(e) The large quantity of plant residues would act as a supply of carbon which is readily rendered available by the soil's rich flora of cellulose ferments. If these soils had produced a wheat crop every alternate year and all of the nitrogen which had been added to the soil without loss from leaching or bacterial activity taken by the crop, it would have necessitated the addition of 25 pounds an acre yearly, which is evidently the very minimum which can be attributed in these soils to non-symbiotic nitrogen-fixation.

Eighty different samples of these soils were incubated in the laboratory for 21 days and the gains in nitrogen determined by comparing with sterile checks. The soils were incubated without the addition of anything except sterile distilled water. At the end of the period the average gain per acre for the cultivated soils was 202 pounds and that for the virgin soil was 92.

True, fixation would not continue long at this rate, for when the nitrogen content of the soil passed beyond a certain limit, decay bacteria increase rapidly (79), and in the struggle for existence they are able, with the advantage at their disposal, to suppress the more slowly growing *Azotobacter*, which would gain the ascendancy again only when the nitrogen of the soil became low.

Thus, there is an upper as well as a lower limit to the nitrogen content of the soil as far as bacterial activity is concerned, but by making the conditions for nitrogen-fixation as nearly ideal as possible we may maintain in a soil the upper and not the lower nitrogen content.

In conclusion, it may be stated that, although the part played by *Azotobacter* in maintaining the nitrogen of the soil has not been definitely measured, it is nevertheless an important factor. Hall (63) found it at least 25 pounds, Löhnis (134) 35.7 pounds, and the Utah Agricultural Experiment Station 25 pounds per acre annually. It is therefore, conservative to state, as has Lipman (125), that these organisms, under favorable conditions, add from 15 to 40 pounds of available nitrogen to each acre of soil yearly.

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